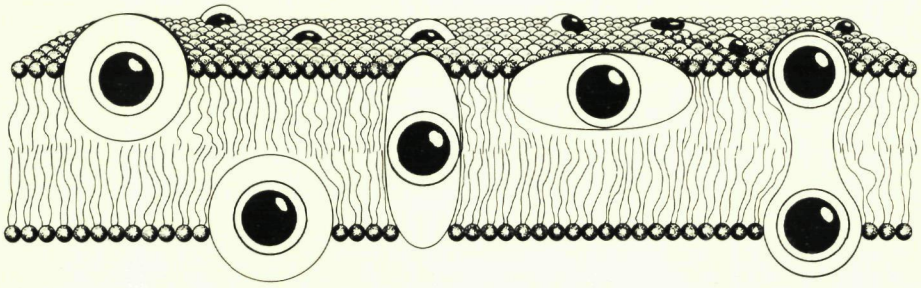
ASPECTS OF LIPID-RHODOPSIN INTERACTIONS IN PHOTORECEPTOR MEMBRANES



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Aan Karine,

Florrie en Gijs

CONTENTS

ABBREVIATIONS

A	absorbance
ATP	adenosine 5'-triphosphate
BHT	2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene)
CTAB	cetyltrimethylammonium bromide
DTAB	dodecyltrimethylammonium bromide
DTE	1,4-dithioerythritol
DTNB	5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent)
EC	Enzyme Classification (Enzyme Handbook; Barman, T.E., ed., Volume I and II, 1969; suppl. I, 1974; Springer Verlag, Berlin)
EDTA	ethylenediaminetetraacetate
GTP	guanosine 5'-triphosphate
HEPES	2-4-(2-hydroxyethyl)-piperazinyl-(1)-ethanesulfonic acid
hr(s)	hour(s)
MW	molecular weight
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced form
OD	optical density (absorbance)
ROS	rod outer segment(s)
SDS	sodium dodecylsulfate (sodium laurylsulfate)
TNBS	2,4,6-trinitrobenzene-1-sulfonic acid
TRIS	tris(hydroxymethyl)aminomethane
ε	molar absorbance coefficient
λ	wavelength
ΔA_{500}	absorbance difference at a particular wavelength (i.c. 500 nm)

PREFACE

During the last century much effort has been expended on the elucidation of the visual process. Apart from studies on intact eyes and isolated retinas, most attention has been paid to the outer segments of retinal rod cells in which the conversion of light into an electrical signal proceeds. The visual pigment rhodopsin must play a key role in this process and it has therefore been extensively studied. Initially these studies were largely confined to the light induced responses of the visual pigment with little reference to its natural location in the photoreceptor membrane. Detergent solubilized preparations were commonly used in order to facilitate spectroscopic techniques. In recent years techniques have become available to study the visual pigment in its membrane environment and it has become increasingly clear that the visual process can only be adequately understood when the effects of rhodopsin illumination on the properties of the photoreceptor membrane are known.

This thesis is mainly concerned with the relations between rhodopsin and its lipid bilayer environment in order to define the requirements for proper functioning of the visual pigment.

In chapter 1 our current knowledge of the rod photoreceptor cell and the assumed role of rhodopsin in the visual process are presented. Chapter 2 deals with our current understanding of the structure and dynamics of the photoreceptor membrane and the interrelations between rhodopsin and its membrane environment. In chapter 3 the general methods and materials used throughout our studies are described. Contributions to the topography of the bovine rod outer segment membrane are presented in chapter 4. It is shown that proteolysis of rhodopsin is limited as a result of partial shielding of the visual pigment molecule by the surrounding lipid bilayer.

Parameters for rhodopsin function are needed for a study of the effects exerted on the visual pigment molecule by changes in its micro-environment. One of these parameters is provided by the late

spectral transitions following illumination of rhodopsin. In chapter 5 this parameter is studied in detail for rhodopsin in its native membrane environment. A partial chemical characterization of the intermediates involved in the transitions is offered. In chapter 6 this parameter is used to study the effects on rhodopsin of partial and complete lipid removal and of changes in the lipid bilayer composition. In chapter 7 the effects of these changes in the microenvironment of rhodopsin are studied by means of two other parameters, viz. the thermal stability of rhodopsin and its regeneration capacity. Chapter 8 presents electronmicroscopic studies of photoreceptor membranes treated with phospholipase C.

Finally, in chapter 9 the main results of the preceding chapters are combined in order to arrive at an overall picture of lipid-rhodopsin relationships. Some suggestions are offered for further studies along the line of this thesis to assess additional aspects of lipid-rhodopsin interactions in photoreceptor membranes and resolve some conflicting results and views in the literature.

CHAPTER 1

STRUCTURE AND FUNCTION OF THE ROD PHOTORECEPTOR CELL

1.1. General morphology

The retina is part of the nervous system and covers the inside of the posterior half of the eyecup. It consists of a thin, about 0.1-0.5 mm thick, layer of tissue, in which incident light, with a wavelength between approximately 400 to 800 nm is converted into nervous signals.

The general pattern of all functionally developed vertebrate retinas appears to be very uniform. Five different cell types, in synaptic contact, can be discriminated: bipolar, amacrine, horizontal, ganglion and photoreceptor cells. The photoreceptor cells reside in a single layer adjacent to the pigment epithelium, and are directly involved in the conversion of light into an electrical impulse. The other cell types mediate and correlate the propagation of the impulses.

Vertebrate retinas generally possess two types of photoreceptor cells: rods and cones. In most species the rods greatly outnumber the cones. In the human retina for instance, only about 7% of the photoreceptor cells are cones, which are highly concentrated in a small spot, the fovea, in the macular area of the retina. A density of about 200,000 photoreceptor cells per mm^2 is found in the human eye (Østerberg, 1935).

The rods and cones are complementary to each other in their function as light receptors. Rods are very sensitive to light and can already be excited by the absorption of a single photon (Hecht et al., 1942; Bouman and Van der Velden, 1947). They operate primarily in twilight (scotopic vision), and since rods contain only one type of visual pigment, they can discriminate light intensities, but not

colours (black-white vision). Cones have a threshold for light, which is much higher than that of rods. Cones therefore function under daylight conditions (photopic vision, cf. Dartnall, 1972; Brown, 1973; Werblin, 1973). Three types of cones may generally be discriminated, each characterized by a visual pigment of different spectral sensitivity, mediating colour vision in a mosaic pattern (cf. Richards, 1975; Wooten and Wald, 1973).

Rods and cones are elongated cells, axially oriented to the incident light, and consist of two parts, the inner segment and the outer segment. These segments are connected by a non-motile cilium (fig. 1.1). The cells derive their names from the cylindrical and

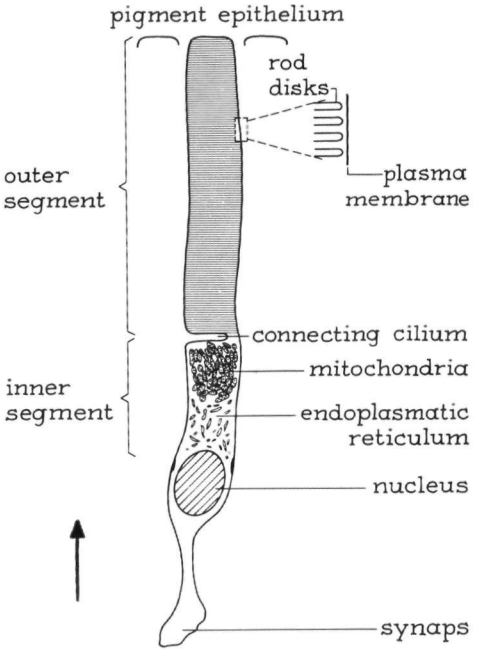


Fig. 1.1. Schematic diagram of the rod photoreceptor cell. The direction of the incident light is indicated by the arrow. Modified after Young (1971).

conical shapes of their respective outer segments. The common intracellular organelles - nucleus, Golgi apparatus, mitochondria and endoplasmic reticulum - are located exclusively in the inner segment. This implies that biosynthetic and bioenergetic products, required by the outer segment, must pass through the connecting cilium (Young, 1968, 1976). Single ciliary microtubules extend far into the outer segments, while clefts are frequently observed (Steinberg, 1973, Steinberg and Wood, 1975).

1.2. Outer segment structure

The outer segments of the photoreceptor cells have a characteristic structure. Rod as well as cone outer segments contain a pile of hundreds of flat plan-parallel membranes, which are oriented perpendicular to the long axis of the cells and thus to the direction of the incident light (fig. 1.1).

In cones the plasma membrane is heavily invaginated over the entire length of the outer segment, so that the pile of parallel membrane sheets is formed by a single continuous membrane (fig. 1.2).

In rods, however, the plan-parallel membranes form closed, flat sacs, which are not connected to the surrounding plasma membrane (fig. 1.2 and 1.3). Since these sacs on superficial electron-microscopic observation appear as solid discs, the term disc (disk) is frequently used. The discs are formed by invagination of the plasma membrane at the basal part of the rod outer segment, close to the connecting cilium, where the rod outer segment resembles the cone structure (fig. 1.2). The invaginated regions are subsequently pinched off. This means that in this process the outer face of the plasma membrane inverts and becomes the inner face of the rod disc membrane.

In bovine retinas,used throughout our studies, rods greatly outnumber cones. Thus,mass isolation of outer segments in essence yields a rod outer segment preparation, and hence we shall confine our further discussion to rod outer segments.

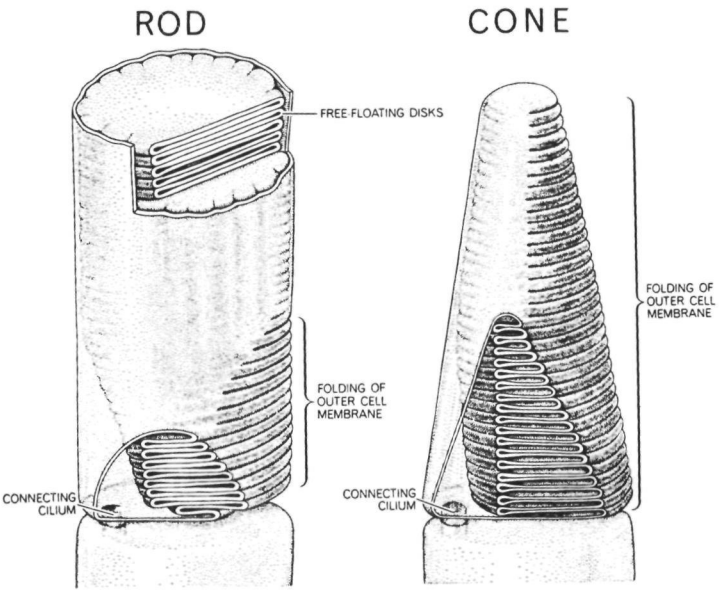


Fig. 1.2. Schematic presentation of the rod and cone outer segment. The rod outer segment has been cut to reveal the pile of free-floating discs surrounded by the plasma membrane (outer cell membrane). The basal part is opened to show the region of the invaginated plasma membrane, where the discs have not yet been pinched-off. The opening in the cone outer segment discloses the pile of parallel membrane sheets, which are all part of one continuous (plasma) membrane. The number of discs and invaginations has greatly been reduced for simplicity. The rod outer segment is commonly at least 3 times longer than the cone outer segment. From Young (1970).

The dimensions of the rod outer segment are typically 50 μ x 6 μ in frog, 24 μ x 1.7 μ in rat and 20 μ x 1 μ in cattle and the number of discs within one outer segment varies from about 500 to 2000. Under conditions which may optimally reflect the native situation an intradiscal space is seen with a wideness of about 20 Å (Nir and Pease, 1973; Chabre and Cavaggioni, 1975). Ultrastructural studies have revealed that rod discs respond to osmotic changes (Heller et al., 1971; Cohen, 1971; Falk and Fatt, 1973). X-ray diffraction studies also indicate that they may act as osmometers (Chabre and Cavaggioni, 1975). Nevertheless, it is not unambiguously

settled whether the intradiscal space consists of a fluid or rigid matrix or whether the discal membrane is internally fused, leaving no room for an intradiscal space (Kessler et al., 1974).

The disc-to-disc repeat distance, although influenced by osmotic strength and ionic composition of medium surrounding the outer segment (Korenbrot, 1973), is highly uniform over a great length of rod outer segment (cf. fig. 1.3), and normally amounts to about 300 Å (Korenbrot et al., 1973; Chabre, 1975).

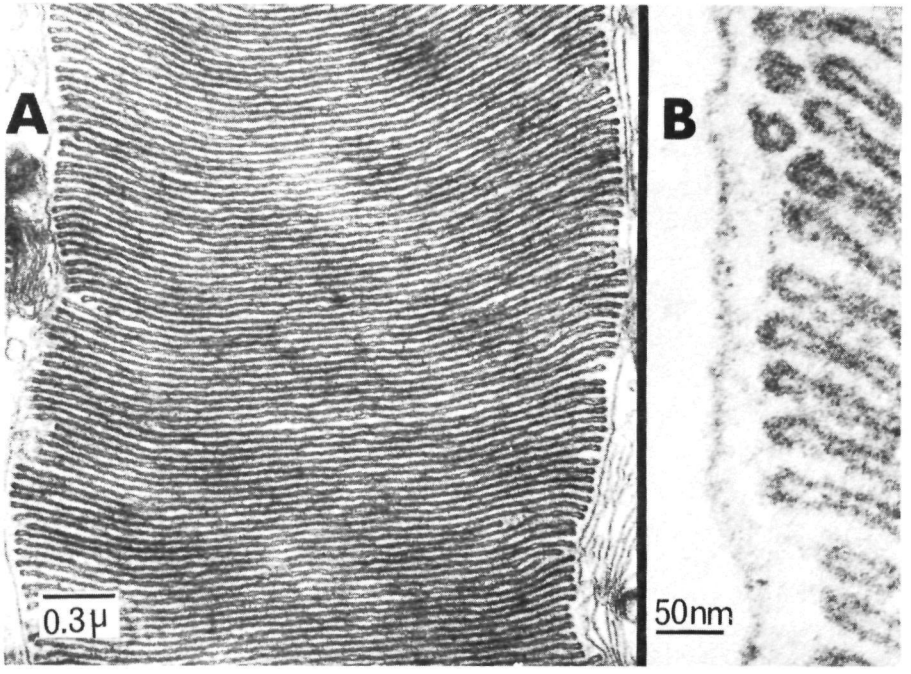


Fig. 1.3. Electronmicrograph of a longitudinal section of part of a bovine rod outer segment, showing (A) the pile of regularly stacked discs, which (B) are not in contact with the plasma membrane. Small intradiscal spaces can be observed, which are somewhat more pronounced at the sharply bent edges of the discs.

The regular arrangement of the discal membranes facilitates the application of physical techniques, which demand an uniform lattice. The rod outer segments have magnetic anisotropy (Chalazonitis et al., 1970; Chagneux-Costa , 1975), which is explained on the basis of the plan-parallel alignment of the membranes (Hong et al., 1971). Application of a magnetic field causes parallel orientation of rod outer segments in an aqueous environment, which has been utilized in X-ray studies (Chabre, 1975; Chabre and Cavaggioni, 1975) and neutron diffraction measurements (Saibil et al., 1976).

1.3. Rod outer segment isolation and composition

1.3.1. Isolation

When isolated retinas are shaken or mildly homogenized, the outer segments tend to break off at the ciliar connection. The low density of rod outer segments (1.08-1.10), as compared to that of other cell organelles, enables isolation of pure rod outer segments by means of density gradient centrifugation (De Grip et al., 1972; Papermaster and Dreyer, 1974). Suspensions of free photoreceptor membranes can be obtained through lysis of isolated rod outer segments by strong hypotonic treatment and washing. This results in vesicular membrane structures (cf. fig. 4.3) and removal of soluble components, leaving a pure membrane preparation. Rod outer segments isolated in darkness, have a red-purple colour, which quickly changes (bleaches) to pale yellow in the light. The characteristic colour is due to the visual pigment rhodopsin.

The rod outer segment membrane is one of the most extensively studied biomembranes due to the relative ease of large scale isolation, the presence of a natural marker (rhodopsin) and the great structural and functional similarity between these membranes in different species. Cattle and frog photoreceptor membranes have been used most commonly.

1.3.2. Lipids

On a dry weight basis, rod outer segment membranes contain about 60% lipids and 40% protein. The main protein component of these membranes is the visual pigment rhodopsin (see section 1.4), which accounts for 80-90% of the total protein content of the photoreceptor membrane (Hall et al., 1969; Bownds et al., 1971; Robinson et al., 1972; Heitzmann, 1972; Daemen et al., 1972; Papermaster and Dreyer, 1974).

Over 80% of the lipids in the rod outer segment membrane are phospholipids, while cholesterol accounts for 6% of less and glycolipids are hardly present (cf. Daemen, 1973; Edel-Harth et al., 1973; De Grip, 1974). The molar ratio between phospholipids and rhodopsin is about 65. The total lipid composition of cattle, frog and rat outer segments appears to be very similar (cf. Daemen, 1973). The average percentages for the three predominant phospholipids are listed in table 1.1. Sphingomyelin and phosphatidylinositol account

Table 1.1. ROD OUTER SEGMENT MEMBRANE COMPOSITION

% of dry weight					
Protein	40 %	of which rhodopsin is >85%			
Phospholipid	50 %	phosphatidylcholine	44 %	fatty acids	$C_{16:0}$ 12%
		phosphatidylethanolamine	39 %		$C_{18:0}$ 18%
		phosphatidylserine	14 %		$C_{22:6}$ 50%
Cholesterol	3 %				
% of wet weight					
Rhodopsin	10 %	(2.5-3.5 mM)			
Phospholipids	15 %	(100-200 mM)			

for only a few percent. The structure of the most common glycerophospholipids is shown in fig. 1.4.

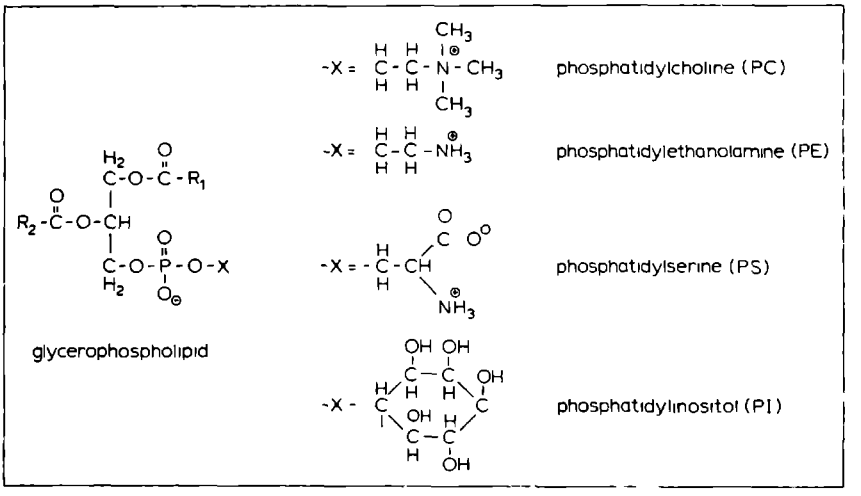


Fig. 1.4. Structure of four most common glycerophospholipids. R_1 and R_2 denote (different) hydrocarbon chains.

While the phospholipid composition is not strikingly different from that of other biomembranes (cf. Daemen, 1973), the high degree of unsaturation of their fatty acid chains (more than 60% unsaturated), is rather unique. The dominant fatty acid (37 mole %) is the long chain polyunsaturated fatty acid, docosahexaenoic acid (C22 : 6 ω 3) (Borggreven et al., 1970; Anderson and Maude, 1970).

The level of unsaturation in the phospholipids increases somewhat in the order: phosphatidylinositol < phosphatidylcholine < phosphatidylethanolamine < phosphatidylserine. The last phospholipid hardly contains palmitic acid (C16 · 0), while in the others it accounts for at least 15% of the fatty acids. The unsaturated fatty acids are predominantly linked to the 2-position in all glycerophospholipids of the rod outer segment membranes (Anderson and Sperling, 1971; Anderson and Risk, 1974). Only phosphatidylserine of cattle photoreceptor membranes contains an appreciable amount (∿ 10%) of C22 : 4 and C22 : 5 fatty acids, attached to the 1-position of the glycerol backbone (Anderson et al., 1975).

The importance of the high level of unsaturation is illustrated by dietary experiments. The outer segments tend to retain their original fatty acid composition much longer than other tissues, when rats are fed a diet deficient in essential fatty acids (Futterman et al., 1971; Anderson and Maude, 1972). Nevertheless, a reduction in unsaturation is achieved when the diet is continued for long periods or during several generations, especially the amount of docosahexaenoic acid is then appreciably lowered. This hardly influences the visual pigment concentration, but the light-evoked electric signals (electroretinogram, ERG) of the retinas are altered in comparison to controls. Also differences in the rate of biosynthesis (renewal) of the photoreceptor membranes have been observed (Benolken et al., 1973; Landis et al., 1973; Anderson et al., 1974, 1976; Wheeler et al., 1975, Dudley et al., 1975).

If rod outer segment membranes are illuminated, production of radicals may occur. This can lead to oxidation of the fatty acid chains (Kagan et al., 1973, 1975, Pontus and Delmelle, 1973; Delmelle and Pontus, 1975). High concentrations of vitamin E (α-tocopherol) in the photoreceptor membranes (Fransworth and Dratz, 1976), the presence of a superoxide dismutase (Hall and Hall, 1975) and a high turnover of fatty acids (Bibb and Young, 1974a) are likely to serve prevention of oxidative damage of the fatty acid chains.

The persistent preservation of the amount of unsaturated phospholipids, the modified responses of the retina after essential fatty acid deprivation and the safeguards against lipid-peroxidation present in the outer segments are all suggestive for a functional relation between the level of unsaturation and photoreceptor operation. A molecular explanation of this relationship does not yet exist (compare chapter 9).

1.3.3. Enzyme activities and proteins

Various enzyme activities have been observed in rod outer segments. Retinoldehydrogenase (retinol oxido-reductase) converts

retinol to retinaldehyde, or vice versa, in the presence of appropriate coenzymes (Bridges, 1962; Futterman, 1963; De Pont et al., 1970a; Kissun et al., 1972). The enzyme is virtually specific for the all-trans isomers of retinaldehyde and retinol (Lion et al., 1975; Lion, 1976) and is intrinsic to the photoreceptor membrane. The enzyme is an entity distinct from rhodopsin, because it can be separated from the latter by preferential extraction with non-ionic detergent (Etingof et al., 1972). In retinoldehydrogenase an aminogroup plays an essential role in the binding of the substrate and a specific sulfhydryl group appears to be necessary for the binding of the coenzyme (De Grip et al., 1975b).

Esterase and isomerase activities are also postulated to be present, since there is evidence for hydrolysis of retinyl-esters and isomerization of all-trans retinaldehyde (or derivative) to the 11-cis form in the outer segments (cf. Bridges, 1976a and b).

The membrane bound (Na^+-K^+)-ATPase has been reported to be present in rod outer segment preparations (Bonting et al., 1964, Etingof et al., 1972; Hemminki, 1974). While this activity may be largely due to contamination with membrane structures possibly derived from the inner segment, the presence of some activity in the outer segment cannot be completely excluded (Hendriks, 1975; Zimmerman et al., 1976). Despite the observation that the ATP concentration in rod outer segments is strongly influenced by illumination (Caretta and Cavaggioni, 1976), the existence of a calcium specific ATPase (Ostwald and Heller, 1972) is doubtful (cf. Bonting and Daemen, 1976).

Enzymes of cyclic nucleotide metabolism have also been detected. Adenylate cyclase activity is present (Bitensky et al., 1971, 1972), but the claim for its light sensitivity and unusually high activity could not be confirmed (Hendriks et al., 1973; Bownds et al., 1974; Manthorpe and McConnell, 1974; Zimmerman et al., 1976) and has partially been withdrawn (Miki et al., 1973, 1974; Bitensky, 1973). A role of cyclic AMP in the recovery of photoreceptor sensitivity after illumination is suggested (Bownds et al., 1974), but not generally accepted (Hood and Ebrey, 1974). Several authors have

reported the presence of a guanylate cyclase activity in rod outer segments (Klethi et al., 1970; Pannbacker, 1973, 1974; Goridis et al., 1973), which is light sensitive under specific conditions (Goridis et al., 1973; Bensinger et al., 1974). This enzyme appears to be an intrinsic component of outer segments (Schmidt and Lolley, 1973; Zimmerman et al., 1976; Virmaux et al., 1976). Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase may be seen as antagonists of the cyclase activities, and they are generally studied in combination. The presence of both esterases in rod outer segments has been reported (Pannbacker et al., 1972; Pannbacker, 1974; Chader et al., 1974; Goridis et al., 1974) and their activities seem to be increased by illumination (Miki et al., 1973 , 1975; Goridis and Virmaux, 1974; Manthorpe and McConnell, 1975). Phosphodiesterase activity has also been detected histochemically (Robb, 1974a and b), but only the cyclic GMP phosphodiesterase apparently indeed resides in the rod outer segments (Coquil et al., 1975; Zimmerman et al., 1976). Cyclic GMP has been reported to be present in high concentrations (Krishna et al., 1975). Its role in photoreceptor function is not yet understood, although a role in light and dark adaptation has been proposed (cf. Bensinger and Podos, 1975; Bordie and Bownds, 1976; Goridis et al., 1976).

Rhodopsin or its photolytic products may be phosphorylated by a protein kinase (Kühn and Dreyer, 1972; Bownds et al., 1972; Chader et al., 1975). This protein kinase is water-soluble, but is inherent to the rod outer segments (Frank and Bensinger, 1974; Weller et al., 1975c), and appears to operate in vivo (Kuhn, 1974). Illumination leads to a drastic increase in phosphorylation, but it is not clear which of the rhodopsin photolytic products is the actual substrate (Paulsen et al., 1975; Miller and Paulsen, 1975; Kuhn and Bader, 1976). Other proteins may also act as substrate for the protein kinase,questioning the latter's specificity (Frank et al., 1973; Frank and Bensinger, 1974; Kuhn et al., 1973; Frank and Buzney, 1975), while the stoichiometry of the reaction is in dispute (Bownds et al., 1972; Shichi et al., 1974). Two distinct protein kinases may be involved, one utilizing ATP, the other GTP

(Chader et al., 1976). The rate of phosphorylation seems to exclude a direct role of the kinase in the photoreceptor excitation mechanism (Kuhn and Bader, 1976), but it has been suggested that phosphorylation plays a role in light and dark adaptation (Miller et al., 1975; Weller et al., 1975a and b).

Other enzymes which may be present in rod outer segments are superoxide dismutase (Hall and Hall, 1975) and γ-glutamyltranspeptidase (Tate et al., 1976). Low levels of still other enzyme activities may also occur (Zimmerman et al., 1975, 1976; Yates et al., 1976). Retinol binding proteins may be involved in the transport of retinol or retinyl-esters to or from the pigment epithelium, since their presence in rod outer segments has been reported (Heller, 1976).

1.3.4. Calcium

Lately, the concentration and localization in the rod outer segments of the calcium ion has intensively been studied in view of its alleged role as a transmitter, linking light-induced events at the discal membrane to permeability changes in the plasma membrane (Yoshikami and Hagins, 1971, Hagins, 1972). As will be discussed in section 1.5, this hypothesis requires a localization of Ca^{2+} at or in the discs. Since the isolation procedure may easily influence the ionic composition, the values reported for the Ca^{2+} content of rod outer segments range from 1 to over 10 mol Ca^{2+}/mol rhodopsin (Liebman, 1974; Hendriks et al., 1974; Hagins and Yoshikami, 1975; Hess, 1975; Yoshikami and Hagins, 1976; Farber and Lolley, 1976). Experiments of Hendriks et al. (1974) and Schnetkamp (cf. Bonting and Daemen, 1976) indicate that calcium is mainly present within the discs.

1.4. Rhodopsin

1.4.1. Spectral characterization

This section will be confined to rhodopsin as the visual pigment and will mostly deal with the opsin-chromophore relationships, while chapter 2 will deal with rhodopsin as a membrane protein and discuss rhodopsin-lipid relationships.

The visual pigment rhodopsin consists of a protein, called opsin, and a chromophoric group, 11-cis retinaldehyde. It has a typical absorption spectrum, shown in fig. 1.5, for bovine rod outer

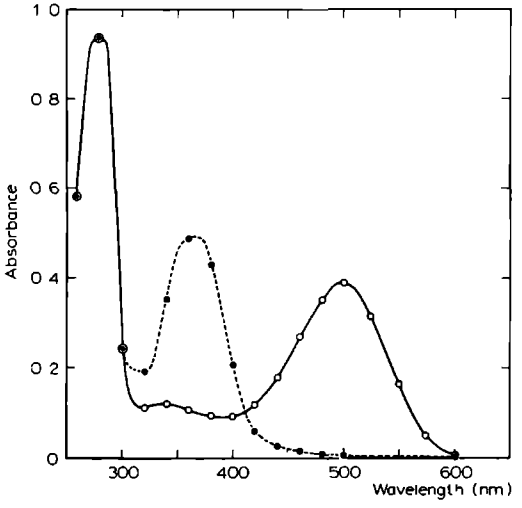


Fig. 1.5. Absorption spectrum of rod photoreceptor membranes solubilized in 1% digitonin solution, before (solid line) and after (dashed line) illumination. The presence of hydroxylamine during this light-exposure led to the formation of retinylidene oxime (Re-C=N-OH, λ_{max} = 365 nm).
H

segment membranes solubilized by digitonin. Three main absorption bands are apparent with maximal absorbance at respectively 500 nm (α-band), 340 nm (β-band) and 278 nm (γ-band). The absorption maximum at 278 nm is common for proteins and is due to the aromatic

rings of tryptophan and tyrosine. The α- and β-bands are characteristic for rhodopsin. Illumination with light of wavelengths around 500 nm causes the disappearance of both bands. They are ultimately replaced by a new absorption band with λ_{max} = 380 nm, which represents all-trans retinaldehyde liberated from opsin. Addition of hydroxylamine speeds up and completes the bleaching process, with a shift of the 380 nm maximum towards about 365 nm as a result of oxime formation with retinaldehyde (cf. fig. 1.5). Hydroxylamine does not affect rhodopsin prior to illumination, hence does not have access to the chromophoric centre of rhodopsin. The difference in absorbance at 500 nm before and after illumination (ΔA_{500}) is a direct measure for the rhodopsin concentration. By means of the molar absorbance of 40,500 at 500 nm (Wald and Brown, 1953; Daemen et al., 1972), the ΔA_{500} value can immediately be converted into a molar rhodopsin concentration.

The rhodopsins of different vertebrate species show variations in absorption maxima between 485 and 515 nm (e.g. Lythgoe, 1972), while small differences between individuals of a certain species may be present (Bowmaker et al., 1975). For cattle rhodopsin, used throughout our studies the absorption maximum is very close to 500 nm. Direct evidence for the involvement of rhodopsin in light perception is provided by the observation that the spectral sensitivity of the human rod system matches exactly the α-peak of rhodopsin (Lythgoe, 1937; Chase and Haig, 1938; Wald, 1945; Crescitelli and Dartnall, 1953; Wald and Brown, 1958).

Rhodopsin has a molecular weight of about 35,000 - 39,000 (Hubbard, 1954; Robinson et al., 1972; Heitzmann, 1972; Daemen et al., 1972; Lewis et al., 1974; Frank and Rodbard, 1975). It is a glycoprotein (Heller, 1968a) and, at least in cattle, the carbohydrates consist exclusively of mannose and glucosamine (Heller and Lawrence, 1970; Plantner and Kean, 1976).

All visual pigments owe their spectral characteristics to the specific combination of a protein combined with one of two forms of vitamin A aldehyde: retinaldehyde or 3-dehydroretinaldehyde (cf. fig. 1.6). Retinaldehyde is a polyenic molecule, which can exist in

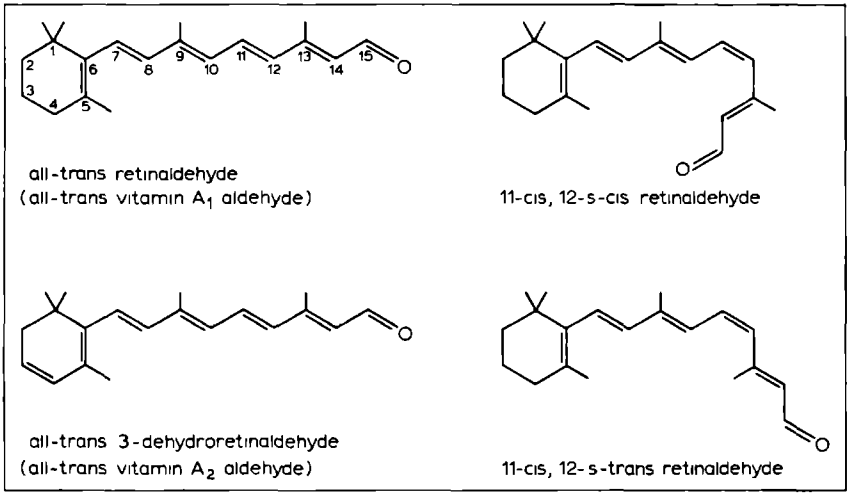


Fig. 1.6. Structure of chromophores.

various cis-trans stereo-isomers. Only the 11-cis isomer (fig. 1.6), which is very unstable in the free form, is found in rhodopsin (Hubbard and Wald, 1952a, 1952b; Rotmans et al., 1972; Rotmans, 1973). The exact conformation of 11-cis retinaldehyde within rhodopsin is still not quite settled. This is due to uncertainties about the precise orientation of the single bonds, about which rotations are possible in free solution, but probably not within the protein (Honig and Ebrey, 1974; Ebrey and Honig, 1975). Recent Raman spectroscopic observations indicate a (possibly distorted) 11-cis, 12-s-trans configuration (Callender et al., 1976; cf. fig. 1.6).

In native rhodopsin retinaldehyde is linked to an amino group by an aldimine band (Collins, 1953; Morton and Pitt, 1957; Rimai et

al., 1970). This so-called Schiff base is protonated at the nitrogen atom as shown by Raman spectroscopy (Lewis et al., 1973; Oseroff and Callender, 1974; Mathies et al., 1976; Callender et al., 1976). Removal of all amino group bearing phospholipids is possible without change in 500 nm absorbance, which indicates that the chromophore is bound to opsin (Borggreven et al., 1972). It has been shown unambiguously that the chromophore is linked to the ε-amino group of a specific lysine residue (Fager et al., 1972; De Grip et al., 1973b, c, d). The possibility that a sulfhydryl group is also directly involved in the binding of the chromophore has recently been excluded by the modification of all free SH-groups by methylmercuric iodide without appreciable loss of 500 nm absorbance (Daemen et al., 1976).

Agents like sodium borohydride and hydroxylamine, which easily react with solubilized retinaldehyde or its Schiff bases (see fig. 1.7), do not attack the aldimine bond in rhodopsin (Bownds and Wald,

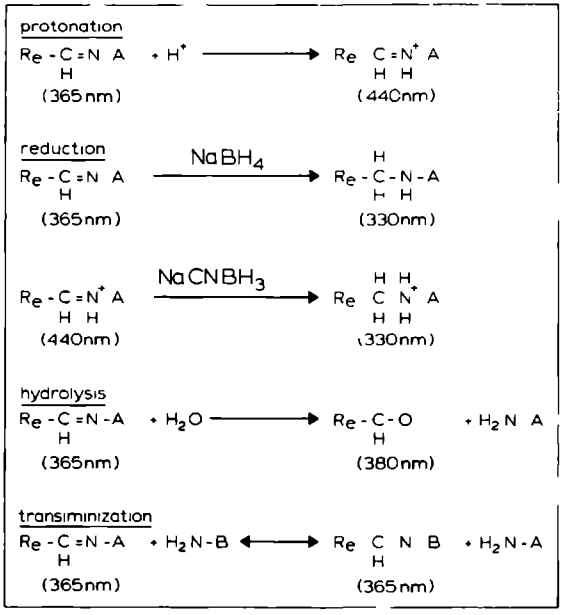


Fig. 1.7. Reactions of Schiff bases of retinaldehyde (retinylidene imines).

1965). Only cyanoborohydride is capable of slowly reducing the aldimine bond (see fig. 1.7) in detergent solubilized rhodopsin (Fager et al., 1972), but not when rhodopsin resides in the intact membrane. Lipoxygenase in the presence of linoleic acid easily leads to oxidation of the polyene chain of free retinaldehyde, but does not affect the chromophore when it is in rhodopsin (Wald and Hubbard, 1960), as we have confirmed (unpublished results). These observations indicate that the chromophore is completely enclosed within the protein.

In detergent solution or alcohol, the maxima for free retinaldehyde and its protonated and unprotonated Schiff bases (aldimines, retinylidene imines) are about 380, 440 and 365 nm, respectively (De Pont et al., 1970b; Daemen et al., 1971). The large bathochromic shift of rhodopsin relative to the protonated Schiff base of retinaldehyde must be due to certain specific interactions of the protein with the polyenic chromophore. Several suggestions, based on theoretical calculations, have been advanced to account for this bathochromic shift, but so far none of them is supported by direct experimental evidence (cf. Honig and Ebrey, 1974; Ebrey and Honig, 1975; Honig et al., 1976).

There are several indications that only 11-cis retinaldehyde fits optimally within the opsin molecule. A few 11-cis isomers of structurally modified retinaldehyde can form photolabile pigments with opsin (Bridges, 1967; Kropf et al., 1974; Ebrey et al., 1975; Kropf, 1976). Most mono-cis and several di-cis isomers of normal retinaldehyde, but not all-trans and 13-cis retinaldehyde, have the same capability (Crouch et al., 1975; Ebrey et al., 1975; De Grip et al., 1976; Crouch, 1976). Such artificial pigments are, however, less easily formed in the reaction analogous to that of 11-cis retinaldehyde with opsin and are in addition less stable than rhodopsin. Only iso-rhodopsin (9-cis retinaldehyde + opsin) is hardly inferior to rhodopsin, but it has like nearly all artificial pigments a less pronounced bathochromic shift. These observations indicate that 11-cis retinaldehyde interacts most extensively with the opsin molecule.

1.4.3. Photolysis of rhodopsin

The primary action of light can be described as the catalysis of a cis-trans isomerization of retinaldehyde with a high quantum efficiency of about 0.6 (Dartnall, 1972). The isomerization is followed (or accompanied) by consecutive spectral transitions, which are all thermal dark-reactions. The intermediates are spectrally defined (cf. fig. 1.8), and photopotentials (early and late receptor potentials) have been correlated with the spectral observations (cf. Cone and Cobbs, 1969; Hanawa and Matsuura, 1975).

The early and very labile intermediates bathorhodopsin (prelumirhodopsin) and lumirhodopsin have initially been identified at very low temperatures (cf. Yoshizawa, 1972, Yoshizawa and Horiuchi, 1973; Abrahamson, 1973; Tokunaga et al., 1976), but recently also by ultra-fast techniques in detergent solutions of rhodopsin at physiological temperatures (Busch et al., 1972; Bensasson et al., 1975; Goldschmidt et al., 1976). It is generally agreed that in the metarhodopsin I stage the chromophore has the (strained?) all-trans configuration. The preceding detectable spectral transitions may be interpreted as the mere result of conformational changes in the protein after isomerization of the chromophore, and thus would reflect consecutive changes in the chromophoric environment. The isomerization process would then have to be very fast ($<10^{-12}$ sec) and precede the bathorhodopsin stage. Theoretical calculations and analogy considerations suggest that, despite the short time interval, the actual isomerization may still proceed by a rather complex mechanism (Salem and Bruckmann, 1975; Warshel , 1976), and aromatic groups might also be involved (Shirane, 1975; Alekseev et al., 1976). On the other hand, the entire sequence of spectral events up to metarhodopsin I has been interpreted as reflecting a sequence of intermediates in the isomerization process in which proton transfer and retro-compounds of retinylidene imine would be involved (Kropf, 1969; Van de Meer et al., 1976). Some experimental evidence supports this view (Fransen et al., 1976), but conflicting evidence is also at hand (Kropf, 1976). Nevertheless this interpretation of the photo-

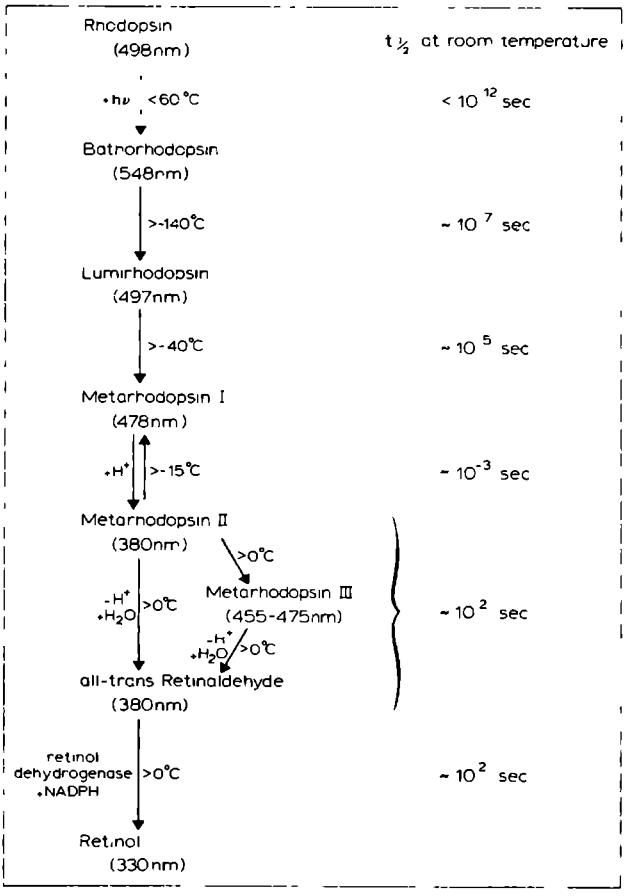


Fig. 1.8. Sequence of intermediates after illumination of vertebrate (bovine) rhodopsin in vitro. The dotted arrow represents the photoreaction. All other arrows indicate thermal dark reactions. The intermediates are characterized by their absorbance maximum (nm). Also indicated are the temperatures above which the reactions can proceed and on the right approximate half-life times at room temperature.

lytic sequence is very attractive, since it would explain the intermediates preceding the metarhodopsin I to metarhodopsin II transition, during which visual excitation is thought to occur.

Through the metarhodopsin II stage the chromophore remains linked to the original lysine residue (Rotmans et al., 1974). From

the hypsochromic shift of the absorption maximum (478 → 380 nm) during the metarhodopsin I → II transition it is concluded that the aldimine group is deprotonated in this step. Strangely, there is simultaneously one proton per pigment molecule taken up from the medium (Radding and Wald, 1956; Falk and Fatt, 1966; Emrich, 1971; Wong and Ostroy, 1973). Metarhodopsin I and metarhodopsin II appear to be in equilibrium with each other, but due to consecutive reactions this equilibrium is never completely reached under physiological conditions. Notwithstanding earlier reports to the contrary, the transition to metarhodopsin II can be described by a single first order process (Applebury et al., 1974; Baumann, 1976). This transition is also accompanied by relatively large conformational changes of the protein (cf. Abrahamson, 1973; Abrahamson and Fager, 1973, Lamola et al., 1974a, 1974b) and possibly by a change of the electric dipole moment of the pigment protein (Petersen and Cone, 1975).

Unprotonated Schiff bases can easily display transiminization (fig. 1.7; Daemen et al., 1971), which is what happens during the decay of metarhodopsin II (Bonting et al., 1973, Rotmans et al., 1974). Metarhodopsin II follows two decay routes (cf. fig. 1.8), namely to opsin plus free retinaldehyde (380 nm) and to metarhodopsin III (pararhodopsin, 455-475 nm), which then in turn decays to opsin plus free retinaldehyde (Matthews et al., 1963; Ebrey, 1968; Cone and Brown, 1969; Weale, 1973). During the thermal decay of metarhodopsin II a proton is released from the protein (Ostroy, 1974). The actual kinetics of the transitions following the metarhodopsin II stage have quantitatively been worked out for frog and human retina on the basis of spectral measurements (Baumann, 1972; Baumann and Bender, 1973; Baumann and Reinheimer, 1973). Quantitative measurements of the metarhodopsin II decay in isolated bovine rod outer segments have never been reported and will be presented in chapter 5, where the chemical nature of metarhodopsin III will be discussed as well.

In vivo or in vitro, in the presence of NADPH, the final form of the chromophore is retinol, resulting from reduction of all-trans retinaldehyde by an all-trans specific retinoldehydrogenase present

in the photoreceptor membrane (Lion et al., 1975). If released in large quantity (exhaustive illumination), retinol may as such or after esterification be transported to the vitamin A stores in the pigment epithelium, which provide the rod outer segments with chromophore for the formation of new visual pigment.

It is not yet clear in which form the chromophore returns to the outer segment, whether as free retinol or its ester, in the all-trans or the 11-cis form. The isomerization of the chromophore to the 11-cis configuration is a crucial step in the visual cycle, but where it takes place and whether as retinol, ester of retinaldehyde is still a matter of dispute (cf. Zimmerman et al., 1974; Lion et al., 1975; Bridges, 1976a and b). Neither is it clear whether an enzyme is involved. There is some evidence for a light-induced isomerization of retinaldehyde as metarhodopsin III or as a protonated aldimine of phosphatidylethanolamine (Shichi and Somers, 1974, 1975), but this cannot explain regeneration in the dark.

1.5. Visual excitation

The principles of the visual excitation process are formulated by Hagins and Yoshikami (1975) as follows: "In all photoreceptor cells that have been studied in sufficient detail, it seems that the photochemical reactions produced by light make themselves felt as sensory responses through a photoelectrical conversion process. That is, the light acts on the cell by controlling the flow of an electric current through its plasma membrane, thus changeing the membrane potential. The membrane potential., in turn, produces an action potential in the photoreceptor cell's axon, ...".

Vertebrate photoreceptors respond to light by decreasing the membrane permeability for sodium ions (Tomita, 1970; Hagins et al., 1970; Korenbrot and Cone, 1972; Chabre and Cavaggioni, 1973). Since there is no direct connection between the photoreceptor membrane and the plasma membrane in rod outer segments, this suggests the intervention of a transmitter substance. Illumination of rhodopsin would

lead to the production or release of a transmitter from the rod disc, which would diffuse to the plasma membrane and there lower the sodium permeability. This leads to a reduction of the sodium current through the plasma membrane.

The light-induced reduction of the sodium current can be imitated by external addition of Ca^{2+}, which has led Hagins to the Ca^{2+} transmitter hypothesis for the excitatory mechanism (cf. Hagins, 1972; Hagins and Yoshikami, 1975; Bonting and Daemen, 1976). The essentials of this hypothesis are illustrated in fig. 1.9. In the

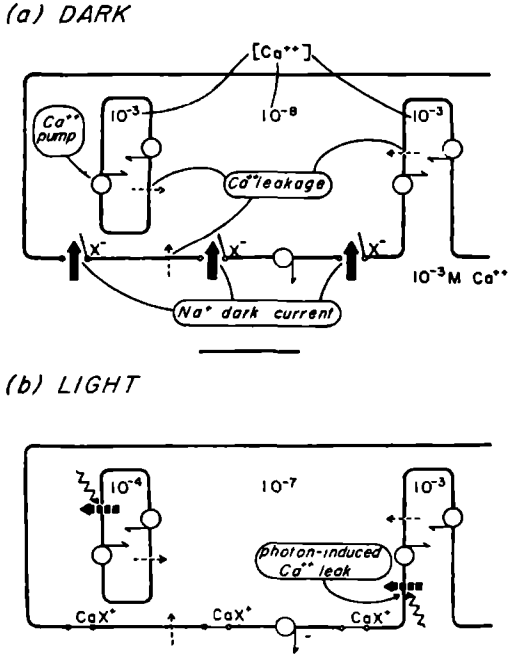


Fig. 1.9. Proposed mechanism for the excitation in vertebrate rods and cones. Values for calcium ion concentrations in cytoplasm and discs do not represent actual measurements. (a) Rod/cone in darkness, (b) rod/cone in the light (Hagins, 1972).

dark adapted state a constant passive Na^+ current is produced by a Na^+-K^+ ATPase pump in the plasma membrane of the inner segment. A

high Ca^{2+} concentration in the discs and a low cytoplasmic Ca^{2+} concentration are maintained in darkness. Upon light stimulation Ca^{2+} is released from the discs and the sudden (local) increase of the cytoplasmic Ca^{2+} concentration leads to a reduction of the sodium permeability of the plasma membrane by closing a number of sodium channels. This results in a hyperpolarization of the plasma membrane, which spreads over the entire membrane and reaches the synapse. This is a quantal process, which can be repeated many times in fast succession until saturation is reached. Eventually calcium must be reabsorbed by the discs in order to activate the rod cell for further stimulation.

The hypothesis has obtained much attention and theoretical considerations show that it may satisfactorily explain the various experimental observations (cf. Hagins, 1972; Hagins and Yoshikami, 1975). Nevertheless an unambiguous proof for the role of Ca^{2+} as the transmitter substance is still not presented. Yet the high Ca^{2+} concentration in the outer segment and its localization at the discal membrane or in the discs support the hypothesis (cf. section 1.3.4). Release of calcium from the discs as a result of illumination has been claimed (Mason et al., 1974b; Liebman, 1974; Herdriks et al., 1974; Weller et al., 1975a), but it is difficult to show that the release is fast enough to reduce the plasma membrane sodium current within milliseconds.

In what way calcium is stored by the discs is not clear, no more than it is known how the actual release of calcium is achieved. One possibility is that the pigment molecule would form an ion channel during the metarhodopsin I → II transition. Hydrogen ion exchange experiments lend some support to this view (Downer and Englander, 1975; Osborne, 1976). Such a mechanism would imply that the rhodopsin molecule has to span the entire width of the photoreceptor membrane, but proof for this is still lacking (cf. section 2.2). Thus, definite conclusions about the precise role of rhodopsin or its photoproducts in the release of the transmitter substance have to await further studies.

In conclusion, it may be stated that the explanation of the

excitatory mechanism has a better theoretical than experimental basis, and thus it still presents a challenging subject for further study. A better understanding of the molecular organization of the photoreceptor membrane may provide important information for the elucidation of the visual process.

CHAPTER 2

THE PHOTORECEPTOR MEMBRANE

2.1. Introduction

In the previous chapter it has been pointed out that the rod outer segment is a highly specialized organelle, designed entirely for its function in the visual excitation process. The characteristics of rhodopsin have there been discussed mainly in relation to its role as visual pigment. The present chapter is directed to structural and dynamic properties of the photoreceptor membrane. Here, we shall discuss the role of rhodopsin as an intrinsic membrane protein, since this thesis is mainly concerned with the relations between rhodopsin and its micro-environment. Although various aspects of vertebrate photoreceptor membranes have been studied in different species (frog, mouse, rat, cattle, monkey and man), no complete picture exists for any single species. Therefore, this survey is presented in an integrated and generalized form.

The rod photoreceptor membranes are organized in a pile of plan-parallel discs and are characterized by the presence of highly unsaturated lipids and an easily detectable natural probe, the visual pigment rhodopsin. This makes them suitable objects for research with all kinds of biochemical and physical techniques. Rhodopsin stands out by its characteristic absorption spectrum, different from that of all other proteins, its light sensitivity and its quantitative dominance over other proteins and high concentration, which is 2.5-3.5 mM in the rod outer segments of various species (Liebman and Entine, 1968; Alpern, 1974; Zwas, 1976). These facts have stimulated the application of various spectroscopic techniques, while

the regular lattice of the stacked discs has favoured the use of X-ray and neutron diffraction. These techniques, together with general methods for membrane study, have revealed several important aspects of the rod outer segment membrane.

In addition to the photoreceptor membrane, the outer segment contains of course a plasma membrane. The latter is important in the generation of a neural signal following illumination of rhodopsin in the photoreceptor membrane (see section 1.5). In addition, through invagination it generates the rod sacs or discs (see section 2.3). The plasma membrane probably also contains rhodopsin (or opsin), as it is shown by immunological experiments (Dewey et al., 1969; Jan and Revel, 1974), lectin binding studies (Molday, 1976; Hall and Nir, 1976) and autoradiography (Basinger et al., 1976a). In view or this, the two membranes may well have a very similar structure and composition. However, the plasma membrane comprises only a small fraction of the total membrane content of the outer segment. In view of the visual pigment orientation in the membrane (see section 2.2.4) only about 10% of the outer membrane is in the optimal position for light absorption, i.e. perpendicular to the direction of the incident light. Thus, the plasma membrane cannot play a significant role in light absorption. Since the photoreceptor (discal) membrane is highly predominant in isolated outer segment membranes, most information has been obtained about this membrane.

2.2. Structural and dynamic properties

2.2.1. Lipid bilayer arrangement

It is generally accepted now that biological membranes consist of a bilayer of amphipathic lipids in association with protein molecules, together forming a complex system of structurally and functionally interrelated components (e.g. Weissmann and Claiborne, 1975). The photoreceptor membrane is no exception in this respect. Electronmicroscopic observations of thin sections show a triple layer image

(cf. fig. 4.3), which is generally found for biomembranes. Intrinsic birefringence of rod outer segments (Schmidt, 1935) is decreased by lipid extraction, which supports a lipid bilayer arrangement (Liebman et al., 1974, Liebman, 1975). Low angle X-ray diffraction studies also confirm this (Worthington, 1974). The diffraction patterns of rod outer segment membranes in situ and in vivo are essentially similar to those of in vitro preparations (Webb, 1972; Schwartz et al., 1975; Santillan and Blasie, 1976). The electron density profile shows at the two extremes a peak of high density, corresponding to the polar head groups of the phospholipids, while protein may also contribute. A central trough of low electron density represents the fatty acid chains (Blasie and Worthington, 1969; Blasie et al., 1969; Blaurock, 1972; Blasie, 1972; Chabre, 1975; Schwartz et al., 1975). The calculated thickness of the rod outer segment membrane is about 70 Å (cf. Corless et al., 1976).

The results of freeze-fracture electronmicroscopic studies of outer segment membranes are also consistent with a lipid bilayer structure, since cleavage is likely to occur preferentially in the plane where the alkyl chains of the two monolayers interact (compare fig. 2.1 and fig. 2.2; cf. Clark and Branton, 1968; Corless et al., 1976).

Some tentative chemical labelling experiments indicate an asymmetric distribution of the phospholipids, with a preference of phosphatidylethanolamine for the cytoplasmic side of the lipid bilayer (Raubach et al., 1974; Litman, 1974).

2.2.2. Size and shape of rhodopsin

Much effort has been spent on the elucidation of the size and shape of rhodopsin molecules, as well as their localization with respect to the lipid bilayer. This information would be very important in relation to the possible role of rhodopsin (or its photolysis products) as an ion-channel. However, considerable disagreement exists about these subjects. Early interpretations of low angle

X-ray analytical data came to a spherical rhodopsin molecule with a diameter of 40-50 Å (Blasie, 1972), but later studies have questioned the validity of these interpretations (Chabre, 1975; Schwartz et al., 1975; Webb, 1976; Blasie, 1976). Electronmicroscopic studies (Blasie et al., 1965; Abrahamson et al., 1974; Nir and Pease, 1975) and gel filtration data of detergent solubilized rhodopsin (Heller, 1968b) also advocate a globular structure.

An elongated, elipsoidal rhodopsin shape (long axis about 70 Å) has been proposed on the basis of proximity relationship measurements with fluorescent probes (Wu and Stryer, 1972; Steinemann et al., 1973), measurements of dichroism and transient electric birefringence of detergent-rhodopsin complexes (Wright et al., 1973; Wright, 1976), X-ray diffraction studies of photoreceptor membranes (Blaurock and Wilkins, 1969;Schwartz and Dratz,1976) and viscosity and turbidity measurements of rod outer segment membrane suspensions (Asai et al., 1975).

In a variant of the elongated structure a separation of protein mass interconnected by β-helical structures has been proposed, the so-called "dumb-bell" model (Poo and Cone, 1973; Sardet et al., 1976). Recently, Raman spectroscopy (Rotschild et al., 1976) has shown that opsin possibly contains α-helix structures, but no β-helix. Neutron scattering analysis indicates a rather close proximity of the centre of mass and a highly asymmetric distribution between hydrophobic and hydrophilic parts of the rhodopsin molecule (Yeager et al., 1976). These results are not in accordance with the suggested "dumb-bell" model, but are more consistent with the three-dimensional model of bacterio-rhodopsin, which includes α-helical segments (Henderson and Uwin, 1975).

2.2.3. Localization of rhodopsin

Depending on the assumed shape of the rhodopsin molecule, all imaginable localizations with respect to the lipid bilayer have been proposed (compare fig. 2.1). The "dumb-bell" model supposes that rhodopsin spans the whole width of the membrane (Poo and Cone, 1973).

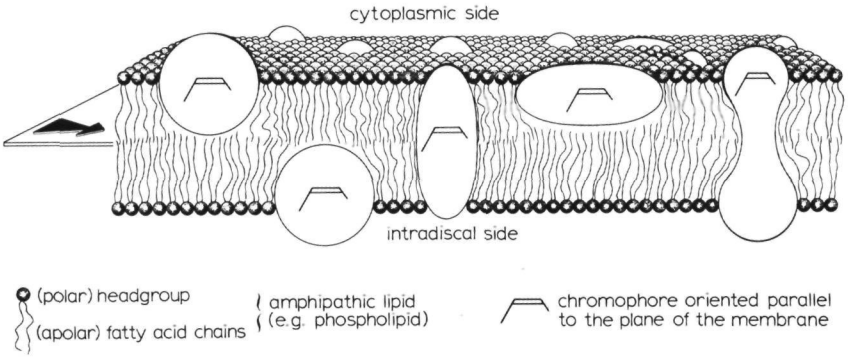


Fig. 2.1. Highly schematic drawing of a lipid bilayer in which rhodopsin molecules are embedded, illustrating some of the proposed shapes and locations of rhodopsin. Spheres located at either side of the membrane, elipsoids with their long axis perpendicular or parallel to the plane of the membrane, and the dumb-bell model are shown. Rhodopsin molecules are drawn in cross-section. The chromophore symbol ( ) is arbitrarily located within the rhodopsin molecules. The white hills represent those parts of the protein molecules, which are exposed to the aqueous phase. The arrow lies in the plane of preferential cleavage during fracturing of deeply frozen specimens. Carbohydrate moieties have not been drawn, since their location is not known for sure.

The elipsoidal model also leaves this possibility, if the long axis of rhodopsin would be perpendicular to the plane of the membrane (Renthal et al., 1973). However, it is also suggested, that this long axis would be oriented parallel to the membrane, ruling out a trans-membrane location (Wright et al., 1973; Asai et al., 1975).

It has also been advocated that rhodopsin is exclusively located at the intradiscal side of the membrane (Gras and Worthington, 1969; Worthington, 1971, 1973) or exclusively at the cytoplasmic side (Blasie, 1972; Corless, 1972; Corless et al., 1976), but models with rhodopsin at both sides of the membrane have also been presented (Blaurock and Wilkins, 1969, 1972; Asai et al., 1975).

The only model which is certainly not correct, is the one in

which rhodopsin is entirely located in the center of the bilayer (Dratz et al., 1972; Dratz and Schwartz, 1973).

The question whether rhodopsin resides in the native membrane as a monomer or in oligomeric form is not yet adequately answered. Energy transfer experiments indicate a monomeric dispersion (Ebrey, 1971a) and from X-ray analysis (Blasie et al., 1965) and calculations from the rhodopsin concentration it is concluded that in that case the molecules would have a mean mutual centre-to-centre distance of about 70 Å (cf. Daemen, 1973). However, this view is not confirmed by freeze-fracture studies (cf. Corless et al., 1976) and rhodopsin cooperativity is suggested on the basis of spectroscopic studies (Robinson, 1975).

Rhodopsin must be partially exposed to the aqueous environment, since it can be attacked by proteolytic enzymes (cf. chapter 4) and it is easily accessible to chemical modification reagents (De Grip et al., 1973b, c, d; De Grip, 1974; Raubach et al., 1974). In addition rhodopsin bears sugar residues (Heller, 1968a), which are presumably exposed to an aqueous phase. Binding studies with the lectin concanavalin A (Steinemann and Stryer, 1973; Renthal et al., 1973; Yariv et al., 1974) and optical polarization studies (Romhanyi and Molnar, 1974) suggest that the sugar residue is located at the cytoplasmic membrane surface, but other binding studies in combination with electronmicroscopic observations indicate an exclusive location of the sugar moieties at the intradiscal surface (Röhlich, 1976). The latter conclusion would also be consistent with the view that the extracellular side of the plasma membrane bears sugar residues (Molday, 1976; Hall and Nir, 1976), which through the invagination of the plasma membrane (cf. section 1.2) would end up at the inside of the disc. In addition, the carbohydrate residues of glycoproteins in all plasma membranes studied so far are always located on the extracellular side (cf. Steck,1974; Bretscher and Raff, 1975). Nevertheless, this still leaves uncertainty about the real location of the sugar residues of rhodopsin.

Electronmicrographs of replicas of freeze-fractured photoreceptor membranes reveal that intramembranous particles reside almost

exclusively at the fracture face of the cytoplasmic half (PF in fig. 2.2) of the rod outer segment membrane (cf. Olive and Benedetti,

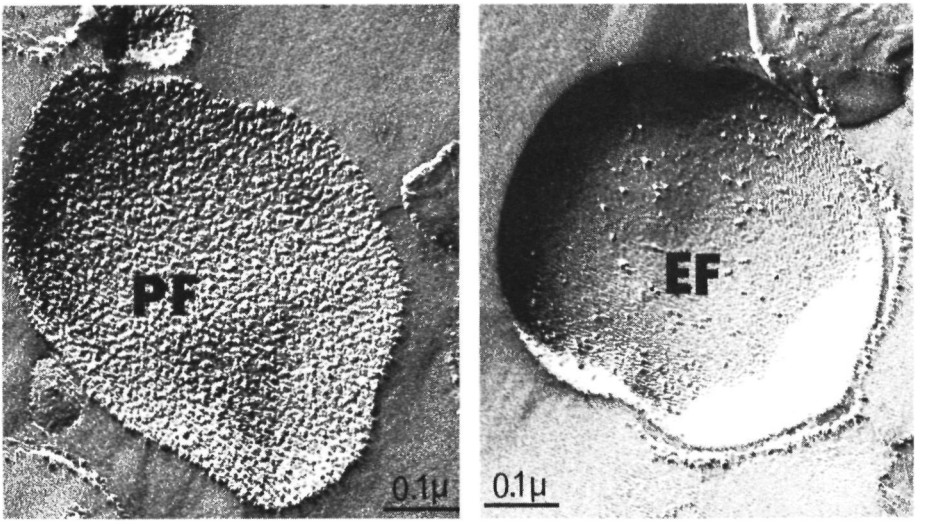


Fig. 2.2. Electronmicrograph of replicas of freeze-fractured rod outer segment membrane vesicles. The particle rich face (PF) represents the fracture face of the cytoplasmic membrane-side. The smooth face (EF) is the fracture face of the intradiscal membrane-side. The symbols PF and EF are those proposed by Branton et al. (1975), see section 8.1.

1974). The mere occurrence of intramembranous particles is no conclusive argument in favour of a trans-membrane location of rhodopsin. It has even been argued that their appearance is artifactual and that rhodopsin does not penetrate into the intradiscal half of the photoreceptor membrane (Corless et al., 1976), although the latter is suggested by results of X-ray diffraction studies (Chabre et al., 1972; Schwartz and Dratz, 1976).

Obviously our current knowledge about the size, shape and location of rhodopsin is far from sufficient to draw a detailed, let alone complete, picture.

More agreement exists about the orientation of chromophore of rhodopsin, 11-cis retinaldehyde. Microspectrophotometric measurements on individual rods have shown an inherent linear dichroism, when they are examined with light perpendicular to the long axis of the rod (side-on illumination and absorbance measurement, cf. fig. 2.3). If this light is polarized parallel to the plane of the

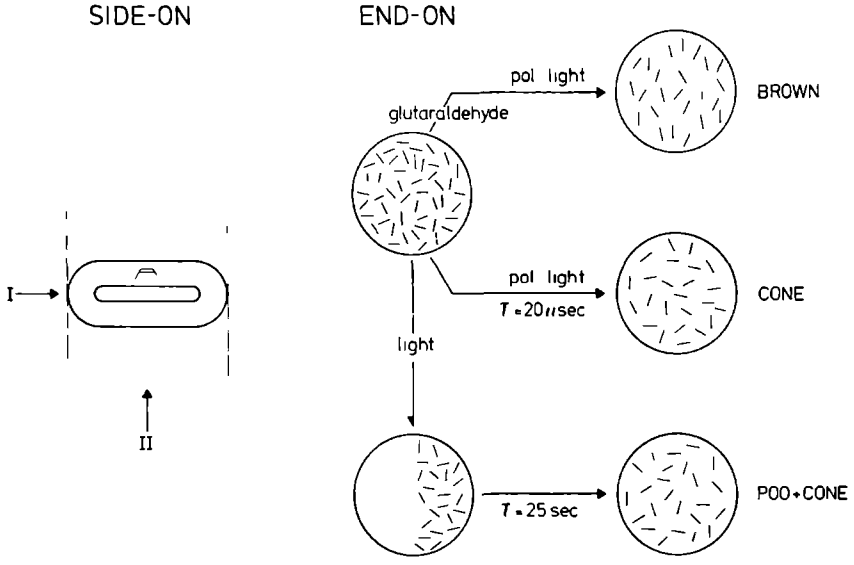


Fig. 2.3. Orientation, rotation and lateral translation of the visual pigment. When viewed from direction I (side-on) the rod outer segments show intrinsic dichroism, implying that all chromophoric groups are continuously oriented parallel to the plane of the membrane. Dichroism is only observed from direction II (end-on) after restriction of molecular motion by glutaraldehyde fixation and subsequent illumination with polarized light (Brown, 1972), or by very fast measurement after illumination with polarized light (Cone, 1972). These observations are indicative for rotational movement of rhodopsin. Lateral movement of rhodopsin is illustrated by the observation that the illuminated half of the photoreceptor membrane becomes refilled with rhodopsin in the absence of rhodopsin regeneration (Poo and Cone, 1973).

discal membranes, the absorbance is greater than with perpendicularly polarized light, yielding a dichroic ratio of 4 to 5 (Schmidt, 1938; Denton, 1959; Hagins and Jennings, 1959, Liebman, 1962; Wald et al., 1963). From these observations it is concluded that the polyene 11-cis retinaldehyde is oriented mainly in parallel to the plane of the membranes. Since it is likely, that 11-cis retinaldehyde closely fits inside opsin and that it cannot rotate within the protein molecule, the results also imply that all rhodopsin molecules are oriented identically. Only at elevated temperatures indications for a loss of this orientation are found (Cone and Brown, 1967, Miljanich et al., 1976).

2.2.5. Rotational motion and lateral diffusion

With classic spectroscopic techniques no linear dichroism is found when native rods are viewed axially (end-on). However, after fixation with glutaraldehyde,end-on illumination with polarized light shows linear dichroism. This observation is explained by assuming preferential isomerization of 11-cis retinaldehyde in those rhodopsin molecules in which the chromophore is oriented parallel to the plane of polarization (compare fig. 2.3). The resulting linear dichroism implies, that rhodopsin molecules in unfixed membranes rotate around an axis perpendicular to the plane of the membrane. A transient dichroism has been observed by means of fast spectroscopic techniques, and a rotational relaxation-time of about 20 µsec is calculated (Cone, 1972).

Apart from this rotational movement, rhodopsin also moves laterally in the membrane (see fig. 2.3). This is shown by an increase in 500 nm absorbance at the illuminated side after lengthwise illumination of one side of a single rod outer segment in the absence of rhodopsin regeneration (Poo and Cone, 1973, 1974, Liebman and Entine, 1974, Agroskin et al., 1975). This implies a diffusion process of rhodopsin molecules within individual discal membranes. However, no rhodopsin appears to be transferred from one disc to the other.

Both the rotational and lateral movements of rhodopsin indicate a great freedom of motion of the pigment molecules. From the rates of movement it is calculated, even in the case of non-spherical rhodopsin molecules (Träuble, 1973) or rhodopsin clusters (Corless et al., 1976), that the lipid phase of the disc membrane is highly fluid with a viscosity of about that of olive oil (Cone, 1972; Liebman and Entine, 1974). This would be consistent with the high degree of unsaturation of the fatty acid chains in the photoreceptor membranes.

Rotational and lateral motion of membrane proteins are not unique for rhodopsin, and similar dynamics are generally accepted for other membranes (cf. Edidin, 1974). However, the rod outer segment membrane has the unique advantage that it has a built-in probe for detecting such movements. This largely excludes the occurrence of possible artifacts from the introduction of extraneous probes.

2.2.6. Phase transition and lateral phase separation

In an aqueous environment the lipids of a lipid bilayer are known to exhibit thermally induced phase transitions between a gel phase and a liquid-crystalline phase (cf. Chapman et al., 1974; Overath et al., 1976). The former phase is found below and the latter above a critical temperature (transition temperature), which is characteristic for any particular kind of amphipatnic lipid. In the gel phase the polar head groups as well as the fatty acid chains are ordered and the latter presumably are all in the all-trans-conformation, while the lateral mobility of the lipids is strongly hampered. In the liquid-crystalline phase the polar head groups retain a fairly regular organization, although they do have considerable mobility. However, the fatty acid chains are in a fluid state and although the hydrocarbon chains maintain an average orientation perpendicular to the plane of the bilayer, they are disordered by rapid trans-gauche rotational isomerizations along the chains. Moreover, in the liquid-crystalline state the lipid molecules undergo rapid lateral diffusion in the plane of the bi-

layer.

In a complex mixture of lipids as is found in most biological membranes, lipids with different transition temperature will be present and it has been observed that a phase separation between lipids in the gel phase and in the liquid-crystalline phase can occur, depending on the temperature of the preparation (Shimshick et al., 1973; Grant et al., 1974, Wu and McConnell, 1975). Areas of ordered lipids and of disordered lipids can be found in a single bilayer and the observation of patches of intramembranous particles in freeze-fractured membranes is frequently interpreted as resulting from lipid phase separations. The intrinsic membrane proteins are thought to reside preferentially in the areas of fluid lipids (James and Branton, 1973; Kleeman et al., 1974, Grant and McConnell, 1974, Kleeman and McConnell, 1974, Haest et al., 1974, Duppel and Dahl, 1976, Wallace et al., 1976).

The various techniques to study these phenomena (e.g. Methods in Enzymology, Vol. XXXII, part B, 1974, Biomembranes, S. Fleischer and L. Packer ed., Academic Press, New York) have also been applied to rod outer segment membranes. It has already been pointed out, that these membranes have a very high level of unsaturation and the finding of a very low transition temperature (Mason and Abrahamson, 1974, Miljanich et al., 1976) is, therefore, not unexpected. The mere presence of rhodopsin in a lipid bilayer seems to decrease the freedom of motion of the lipid chains (Hong and Hubbell, 1972; Hong et al., 1973, Hubbell, 1975) and the fluidity of the photoreceptor membranes may be influenced by the ionic composition of the aqueous phase (Verma et al., 1973) Nevertheless, it seems reasonable that under physiological conditions the majority of the lipids is in the liquid-crystalline phase and hardly any phase separation is expected. Indeed fluorescent probe studies on photoreceptor membranes fail to show any phase separation and a random distribution of particles is observed in replicas of freeze-fractured rod outer segment membranes, quenched by rapid cooling from physiological temperatures (cf. fig. 2.2). Temperature dependent segregation of rhodopsin is only found in reconstituted membranes of rhodopsin and selected

lipids with relatively high transition temperatures, which in this case may be attributed to lipid phase separation (Chen and Hubbell, 1973).

2.2.7. Lipid annulus

Random distribution of proteins does not necessarily imply random distribution of lipids. The structural organization of the lipids surrounding a particular protein may be influenced by the protein involved (Marčelja, 1976). This is suggested by recent experiments with Ca^{2+}-ATPase from sarcoplasmic reticulum, for which it is concluded that the annular lipids are less easily in the ordered state than the rest of the lipid bilayer (Hesketh et al., 1976). Furthermore, in view of the observation that cholesterol is excluded from the lipids surrounding this ATPase (Warren et al., 1975), it seems possible that the composition of annular lipids of integral membrane proteins under physiological conditions differs from the overall composition of the membrane lipids. An annulus of specific lipids or specifically organized lipids may well be essential for appropriate functioning of a membrane protein.

In rod outer segment membranes a similar situation may exist. As pointed out in the previous section, the lipid phase transition in the photoreceptor membrane occurs at low temperatures. Still, a broad thermally induced phase transition is observed at physiological temperatures (Brown et al.,1976a,b) which may indicate the presence of specific lipids around rhodopsin. The mere presence of rhodopsin influences the freedom of motion of the fatty acid chains of a lipid bilayer (cf. Hubbell, 1975), and a separation between rigid and fluid regions has been deduced from electron spin resonance studies on photoreceptor membranes at physiological temperatures (Pontus and Delmelle, 1975a). Furthermore, some tentative observations indicate that phosphatidylserine may be preferentially located around rhodopsin. This phospholipid, which has the highest level of unsaturation of all photoreceptor membrane lipids (Anderson et al., 1975), is most resistent against enzymatic hydrolysis and

extraction with hexane, without denaturation of rhodopsin (Borggreven et al., 1971, 1972).

2.2.8. Effects of illumination

Whereas the elucidation of the structure and dynamics of rod outer segment membranes containing rhodopsin clearly is not simple, even more complications arise if the effects of illumination have to be included. Nevertheless, these effects are highly interesting, since they could possibly reflect changes in the interactions between the membrane components and also reveal features of the mechanism by which rhodopsin illumination leads to a neural signal.

Although much has been published about effects of light on rhodopsin, these effects have unfortunately mostly been studied in detergent solubilized preparations. Thus, they do not necessarily reflect the membrane incorporated state of rhodopsin or its photolytic products, and tell us little about interactions between the membrane components. Therefore, we shall confine us to studies of light effects on intact membranes.

Electron spin resonance measurements suggest a (transient) change in fluidity upon illumination (Delmelle and Pontus, 1975), which probably depends on the ionic composition of the environment (Verma et al., 1973). Birefringence studies reveal a reorientation of a minute number of lipid molecules after illumination (Liebman et al., 1974). Freeze-fracture electron microscopy of artificial membranes containing rhodopsin show light induced changes in phase separation (Chen and Hubbell, 1973), but the results of similar studies on native rod outer segment membranes (Mason et al., 1974a; Abrahamson et al., 1974) are rather doubtful. Rotation and lateral diffusion are probably hardly affected by illumination and natural abundance C^{13}-nuclear magnetic resonance studies (Millett et al., 1974), fluorescent probe measurements (Stubbs et al., 1976b) and circular dichroism studies (Shichi, 1971) do not show significant changes upon illumination. Turbidity and light scattering of photoreceptor membrane suspensions are indicative for only small expan-

sions of the membrane after exposure to light (Asai et al., 1975).

Early X-ray diffraction results were initially interpreted as indicating an inward motion of the visual pigment protein molecule, due to illumination (Blasie, 1972), but this has seriously been contested recently (Chabre, 1975; Chabre and Cavaggioni, 1975). Minor light induced changes of the electron density profile are nevertheless consistently found in the cytoplasmic half of the photoreceptor membrane, although the nature of these changes is hardly understood (Corless, 1972; Chabre, 1975, Chabre and Cavaggioni, 1975). Neutron diffraction studies even suggest an outward shift of the protein as a result of illumination (Saibil et al., 1976). Dichroism measurements have shown that in all photolytic intermediates the chromophore is oriented parallel to the plane of the membrane as it is in rhodopsin (Waggoner and Stryer, 1971; Wright et al., 1973; Kemp, 1973; Harosi, 1975; Tokunage et al., 1976). Bathorhodopsin (Tokunaga et al., 1976) and metarhodopsin III (Harosi, 1975) may show some deviations, but in general one may conclude that the chromophoric environment does not change sufficiently so as to allow rotation of the chromophore. However, as soon as retinaldehyde is reduced to retinol, the latter is preferentially oriented parallel to the alkyl chains of the membrane lipids and thus perpendicular to its original orientation (Kemp, 1973). Apparently only in this stage the opsin molecule imposes no restraints on chromophore movement.

Summarizing, we may say that little is understood of the effects of light on photoreceptor membrane dynamics and the relations between its components. Previously we concluded that the size and shape of rhodopsin are still uncertain and also its location in the lipid bilayer, neither can much be said about the lipid distribution and the effects of light on it. The rotation and lateral diffusion of the rhosopsin molecules are well documented, thanks to the natural probe character of the visual pigment. The orientation of the chromophore parallel to the plane of the membrane in rhodopsin and all its photolytic intermediates is also well established.

2.3. Renewal of photoreceptor membranes

One dynamic aspect of the rod outer segment membrane, the turnover of its components, is still to be discussed. The current state of our knowledge has recently been reviewed by Young (1976). Almost all the evidence is obtained by autoradiographic electron-microscopy, but recently the applicability of the fluorescent-probe technique in this field has been demonstrated (Laties et al., 1976). Various radioactively labeled precursors of different membrane constituents have been used.

There is a continuous process of disc assembly at the base of each rod outer segment, by invagination of the plasma membrane (cf. section 1.2). At the apex,groups of discs are intermittently shed and phagocytized and degraded by the pigment epithelium. Pulse-labeling studies, either with protein precursors or precursor of the oligosaccharide residues of rhodopsin, indicate that discs are gradually moved axially from the base of the rod outer segment to the apex. This continuous process leads to a renewal of all discal membranes of the outer segments in about 10 days in warm-blooded animals and in about 8 weeks in cold-blooded species (Droz, 1963; Young, 1967; Young and Droz, 1968; cf. Young, 1976).

The results of in vitro experiments are consistent with those of in vitro studies (O'Brien, 1972; O'Brien and Muellenberg, 1973, 1974; Basinger and Hall, 1973). The main difference lies in the observation that opsin is synthesized rather than rhodopsin (O'Brien and Muellenberg, 1974, 1975; Papermaster et al., 1975), probably due to lack of sufficient chromophore. The latter is preferentially stored in the pigment epithelium (Zimmerman, 1974; Zimmerman et al., 1974; Bridges, 1976b),which is absent in in vitro experiments.

Outer segments are devoid of RNA (Bok, 1970) and cannot synthesize protein (Young, 1967; Young and Droz, 1968; Hall et al., 1969). The membrane proteins are synthesized in the inner segment of the photoreceptor cell and transported to the outer segment (Young, 1968), presumably by the ciliar membranes as is indicated by in vitro labelling experiments (Papermaster et al., 1975). Rod

outer segments do not take up and incorporate glycerol directly into the membranes (Bibb and Young, 1974b) and enzymes for phospholipid synthesis appear to be largely absent (Swartz and Mitchell, 1970, 1974). Hence, phospholipids, just like proteins, are probably formed in the inner segment. The protein part of the visual pigment (opsin) is complete, at least in terms of its oligosaccharide moiety, when it arrives in the outer segment (Bok et al., 1974). Whether the membrane components are assembled before or at the moment of arrival in the outer segment has not been settled yet (Young, 1974). The incorporation of the chromophoric group (11-cis retinaldehyde) appears to take place after insertion of the newly synthesized opsin molecules in the photoreceptor membrane (Hall and Bok, 1974, 1976).

The pigment epithelium plays an important role in the disc shedding process (cf. Burnside, 1976; Hollyfield, 1976; Anderson and Fisher, 1976; Funahashi et al., 1976; Young, 1976). The process may be influenced by light (La Vail, 1976a and 1976b, Basinger et al., 1976b; Bridges et al., 1976). It is suggested that the disc shedding process is induced either by an increase of phosphatidylcholine content in the apical membranes (Basinger and Hoffman, 1976) or by the attachment of certain carbohydrates to the visual pigment in those membranes (O'Brien, 1976).

Another process of renewal is that of molecular replacement or exchange. The protein constituents, including their sugar moieties, seem to be excluded from this process (Young, 1976). However, fatty acids appear to be replaced all along the outer segment, as shown by pulse-chase experiments. They are rapidly exchanged between pigment epithelium and the inner and outer segments (Bibb and Young, 1974a). The molecular mechanism of the fatty acid exchange is not clear, since hardly any phospholipase activity (Swartz and Mitchell, 1973) or acyltransferase activity (Swartz and Mitchell, 1974) can be detected in outer segments.

The glycerol backbone of the phospholipid molecules of a particular disc is much less subject to replacement. Nevertheless the glycerol label is gradually spread out over other membranes

(Bibb and Young, 1974a). This indicates the existence of a phospholipid exchange mechanism, in which possibly phospholipid exchange proteins are involved. This type of proteins are frequently found in other tissues (Wirtz, 1974, Wirtz et al., 1976). Phospholipid-base exchange reactions,e.g. for serine and ethanolamire, seem to be almost completely absent in rod outer segments (Mizuno, 1976).

As is the case for the fatty acids, the visual pigment chromophore is quickly distributed homogeneously over the rod outer segment (Pourcho and Bernstein, 1975; Hall and Bok, 1976). This is the result of a complex cycle of vitamin A and its derivatives between the pigment epithelium and the (inner and) outer segment, and involves the process of light- and dark-adaptation and biosynthesis.

Summarizing, discs move upward in a rod outer segment as a result of continuous renewal processes. Movement of the visual pigment protein is restricted to diffusion and rotation in the discal membrane (cf. section 2.2.5). A phospholipid exchange process appears to occur and fatty acids are continuously replaced at a high turnover rate. The latter process may function in the maintenance of the high level of unsaturated lipids in the discal membrane. Fatty acid chains, which hve suffered oxidative damage despite protective mechanisms (see section 1.3.2), may thus be rapidly replaced.

2.4. Solubilization, purification and properties of rhodopsin

2.4.1. Solubilization

Rhodopsin is an intrinsic membrane protein since it can only be solubilized spectrally intact by treatment of the photoreceptor membrane with specific aqueous detergent solutions. Concentrations are required which generally completely disturb the bilayer arrangement and lead to micellar solutions of rhodopsin monomers. Several detergents in which rhodopsin maintains its characteristic spectrum are commonly applied for solubilization. Digitonin, a nonionic de-

tergent first introduced by Tansley (1931) is a cholestaneglycoside from Digitalis. This detergent is rather difficult to handle since its solutions are not always stable and may give rise to unexpected precipitations. Nevertheless, it has been extensively used since it is mild in comparison to most other detergents. Of all detergents used so far, it has, in comparison with rhodopsin in the intact membrane, the smallest effects on such rhodopsin characteristics as circular dichroism, thermal stability, kinetics of dark reactions of photolyzed rhodopsin and regeneration capacity. Triton X-100 (Crescitelli, 1967) and Emulphogene BC-720 (Shichi et al., 1969) are also nonionic detergents. The former has the disadvantage of high absorbance at wavelengths under 300 nm. The zwitterionic detergent, dodecyldimethylaminoxide (DDAO; Ebrey, 1971b) is suitable, since it can be removed by dialysis (Applebury et al., 1974). Cetyltrimethylammoniumbromide (CTAB; Bridges, 1957) and dodecyltrimethylammoniumbromide (DTAB) and their homologues are cationic detergents, of which DTAB can also be removed by dialysis (Hong and Hubbell, 1972). Recently dialysable nonionic detergents, alkylglycosides, have been applied to rhodopsin and they also appear to be fairly mild (Stubbs et al., 1976a). Anionic detergents like sodiumdodecylsulfate (SDS) or sodiumdesoxycholate rapidly denature rhodopsin as shown by the loss of its characteristic spectrum at concentrations which solubilize it. Sodium cholate is the only anionic detergent, in which rhodopsin remains fairly stable (Henselman and Cusanovich, 1974, 1976). The absorption spectrum of rhodopsin (see fig. 1.5) is identical in all non-denaturing detergents.

2.4.2. Purification

Separation of rhodopsin from other photoreceptor membrane components may be useful or even necessary for analytical purposes and for reconstitution experiments. It is evidently essential for the preparation of pure rhodopsin suspensions, free of lipids, non-rhodopsin proteins or detergents. In purification of rhodopsin

the application of detergents is inevitable. Density gradients can be used for purification (Osborne et al., 1974), but most procedures are based on chromatographic techniques: ion exchange (Ebrey, 1971b; Osborne et al., 1974), calcium phosphate adsorption, hydroxylapatite adsorption (Bowness, 1959; Shichi et al., 1969; Ebrey, 1971b; Hong and Hubbell, 1972, 1973) or agarose gel permeation (Heller, 1968a; Hall and Bacharach, 1970). Even then complete separation between rhodopsin and other membrane constituents is not in all cases achieved, depending on the detergent used.

An even more selective procedure for rhodopsin purification is based on the observation that concanavalin A, a lectin, binds to the sugar residues of rhodopsin. Therefore, rhodopsin can be subjected to affinity chromatography on columns containing immobilized concanavalin A (Steinemann and Stryer, 1973, Renthal et al., 1973; chapter 3). The choice of the detergent in purification procedures is important, especially with regard to suitability for removal, when this is required for further experimentation.

The A_{280}/A_{500} ratio gives a rough indication of rhodopsin purity, since it will generally be increased by protein contaminations. The lowest values obtained for this ratio are about 1.7, but this is not a guarantee for complete purification. Other methods, like residual lipid determination and electrophoretic protein analysis, are needed to check the rhodopsin purity.

The need to use detergents is a great handicap, since they may have deleterious effects on rhodopsin. This is often revealed wnen the characteristics of the photolytic process of rhodopsin in detergent solution are compared to those of rhodopsin in its native membrane environment. The effects greatly depend on the particular detergent but commonly an increase in the dynamics of the protein is observed. The concomitant destabilization may be attributed to features as size and fluidity of micels and penetration of the detergent into the protein (cf. Helenius and Simons, 1975; Tanford and Reynolds, 1976). One must,therefore, always be careful in applying results of measurements on detergent solubilized rhodopsin to the pigment in its native situation (cf. Daemen, 1974; De Grip, 1974).

2.4.3. Properties of rhodopsin

Rhodopsin of cattle and frog appear to exhibit identical immunological responses (Papermaster et al., 1976), which indicates a great similarity between their opsins in addition to their identical chromophoric group. Most detailed knowledge about the properties of rhodopsin in relation to its membrane environment has been obtained for bovine rhodopsin, due to the relative ease of its large scale isolation.

The amino acid composition of bovine rhodopsin is not exceptional as compared to that of other proteins (Zorn and Futterman, 1971; De Grip, 1974; Plantner and Kean, 1976). The proportion of apolar amino acids, the so-called hydrophobicity index, is over 50%, which is, however, not unusual for intrinsic membrane proteins. The amino acid sequence is only known for a small carbohydrate-containing fragment (Heller and Lawrence, 1970) and for a small fragment containing the lysine residue, which binds the chromophore (Bownds, 1967).

There are probably two distinct carbohydrate moieties linked to asparagine residues, which are 12 amino acid residues apart from each other near the N-terminus of the protein chain (P. Hargrave, private communication). This part of the opsin molecule is rather hydrophilic due to its amino acid composition and the attached carbohydrates (mannose and glucosamine residues). The N-terminus (methionine) is blocked, as is probably also the case for the C-terminus (Albrecht, 1957; Heller, 1968a; De Grip, 1974). The sugar residues appear to be attached to the protein before it is incorporated in the discal membranes (Bok et al., 1974). They may be important for the appropriate insertion of rhodopsin into the discal membrane, while they may also be necessary for the maintenance of the rhodopsin orientation in the lipid bilayer.

Six free sulfhydryl groups are present per molecule of rhodopsin and probably also two S-S bonds (De Grip et al., 1973a; De Grip, 1974). The sulfhydryl groups can be classified on basis of their accessibility for specific SH-group selective reagents (De Grip et

al., 1973a, 1975a).

2.5. Reconstitution of rhodopsin containing lipid bilayers

Reconstitution of membranes is a procedure by which individual membrane components are assembled to form an intact lipid bilayer with or without incorpotated proteins. The components can be brought together in widely varying molar ratios and thus the composition of reconstituted membranes can be predetermined. So, membranes can be made as simple or as complex as is desirable with regard to the parameters to be studied. Comparison between the characteristics of membranes with different compositions may permit conclusions about interrelations and dynamics of the membrane components, as well as about the requirements of certain membrane components for the proper functioning of the intrinsic proteins.

Several procedures have been developed for the reconstitution of membranes (cf. Razin, 1972; Zahler, 1974; Hazelbauer and Changeux, 1974; Racker et al., 1975; Metcalfe, 1975). Techniques have been presented to prepare plan-parallel bilayers (cf. Waldbillig et al., 1976) as well as bilayers (blackfilms) that cover a small perforation in a partition-wall between two aqueous compartments (cf. Mueller et al., 1962; Montal and Mueller, 1972). This "blackfilm" technique enables measurement of various electrical characteristics of the membrane (cf. Ti Tien, 1974). By this technique the membranes can also be prepared with an asymmetric distribution of lipids on both sides of the membrane. The incorporation of functionally operative (large) proteins is hampered by the inevitable use of organic solvents in which these proteins often appear to be insoluble or to become denatured. The preparation of rhodopsin containing "black-films" has been claimed, but the membranes are very unstable and do not allow extensive study (Montal and Korenbrot, 1973). Inappropriate incorporation into the lipid bilayer, as a result of partial structural deformation of the protein, or inadequate solubilization by the organic solvent

(hexane) may be to blame for this.

Reconstitution techniques resulting in vesicular membrane structures are usually based on the preparation of a homogeneous dispersion of selected membrane components, followed by the removal of the dispersive agent, which is either a detergent or a mild organic, water-miscible solvent. The resulting, protein containing lipid bilayers may show all essential membrane characteristics as revealed by methods like freeze-fracture electronmicroscopy, enzyme activity or permeability measurements.

These artificial membranes will commonly show a symmetric distribution of the membrane components over both halves of the newly created membrane. This may be a disadvantage in comparison to natural membranes, since in those membranes the intrinsic protein is preferentially oriented and the lipid asymmetrically distributed (Zwaal et al., 1973; Gordesky, 1976). The importance of lipid asymmetry for proper membrane functioning is hardly understood sofar (cf. Gordesky, 1976), but there is recent evidence for a relation between lipid asymmetry and membrane protein orientation (Haest and Deuticke, 1976).

Orientation of membrane proteins can more readily be interpreted in connection with their specific function, considering the differences in processes at both sides of the membrane. Rhodopsin molecules seem to be identically oriented with respect to the plane of the membrane. This is shown by electronmicroscopic observation of replicas of freeze-fractured discal membranes, where a strong preference of the inner membrane particles (rhodopsin) to remain attached to the cytoplasmic half of the bilayer is noticed (fig. 2.2). The necessity for this identical orientation of all rhodopsin molecules in the photoreceptor membranes is not clear. No function of rhodopsin has yet been found, which would demand such an identical orientation. In reconstituted membranes rhodopsin is randomly oriented, i.e. both freeze-cleaved membrane inner faces show equal particle content (Chen and Hubbell, 1973), but so far no changes in rhodopsin properties has been found, which can be attributed to this fact.

Several reconstitution procedures have been used to obtain rhodopsin-lipid bilayers (Zorn and Futterman, 1971; Chabre et al., 1972; Hong and Hubbell, 1972; Montal and Korenbrot, 1973; Applebury et al., 1974). The best defined procedures are those in which purified lipid-free rhodopsin is combined with amphipathic lipid in a detergent solution. Subsequently the detergent is removed by dialysis (Hong and Hubbell, 1972, 1973; Applebury et al., 1974; Stubbs et al., 1976a). This results in vesicular membranes, provided that the detergent can indeed be completely removed. Yet, one should be aware of possible irreversible deleterious effects of detergents on the protein structure.

The reconstitution procedures offer the possibility to change the micro-environment of rhodopsin either by reassembly with specific lipids or by omission or removal of certain lipids. Analysis of the rhodopsin characteristics may then reveal a functional relation between rhodopsin and its lipid environment.

2.6. Parameters of rhodopsin

Undoubtedly, rhodopsin plays a key role in the visual process and its localization in uniquely organized and structured membranes is essential for its function. A detailed picture of the molecular events between light absorption by rhodopsin and plasma membrane excitation cannot yet be supplied. In order to study the molecular and structural responses of the photoreceptor membrane upon light exposure, it is important to have parameters for rhodopsin which would reflect these responses. The functional operation of rhodopsin in the excitation process, apart from its photolytic process, is still unknown and no chemical parameter for its output signal is available (cf. section 1.5). The changes in the photoreceptor membrane upon illumination may, however, be brought into relation to the interaction of rhodopsin and its membrane environment. Experimental changes in the micro-environment of rhodopsin may exert detectable effects on the properties of rhodopsin. This may give us

insight in the properties of the micro-environment required for proper functioning of the visual pigment. There are at least five parameters, which can be used for this purpose.

The first parameter of rhodopsin is its characteristic absorption spectrum, including its 500 nm absorption. This remains unaffected under a great variety of conditions. It indicates whether the chromophoric centre is unchanged, and thus indicates the intactness of a specific part of the protein. It will not be sensitive enough to reveal effects of alterations in other parts of the molecule.

The second parameter is the thermal stability of rhodopsin, which will depend on the condition of the rhodopsin molecule, and has been found to be useful in this respect (see chapter 7).

A third parameter is the accessibility of specific reactive groups of the protein chain of rhodopsin. Thus far only the sulfhydryl groups have been classified on the basis of their accessibility for specific reagents (De Grip et al., 1975a). If changes in the micro-environment would influence the structural organization of rhodopsin, the accessibility of sulfhydryl groups might change.

A fourth parameter is the regenerability of rhodopsin after photolysis. In vitro incubation of an illuminated rhodopsin preparation, either rod outer segment membrane suspension or detergent solution (Wald and Brown, 1956, Henselman and Cusanovich, 1976) or isolated retinas (Pepperberg et al., 1976), with exogenous 11-cis retinaldehyde leads to formation of rhodopsin. The regeneration capacity (see section 3.3.4), i.e. the extent to which an illuminated preparation can recover its original rhodopsin content by reaction with 11-cis retinaldehyde, and also the rate of regeneration are indicative for structural integrity of opsin and the accessibility of the chromophore binding centre.

A fifth parameter is of course the sequence of thermal dark reactions following rhodopsin illumination, during which conformational changes within the protein are thought to occur (cf. section 1.4.3). Alterations in the formation and decay of the intermediates

and the kinetics of the transitions may give information about changes in the protein.

These parameters are all intrinsic parameters of rhodopsin, which do not require potentially artifactual manipulations, such as the introduction of probes. They can all be measured in membrane suspensions and can be studied in combination.

2.7. Aims of this study

The major purpose of this study is to increase our understanding of the relationships between the major membrane constituents of the rod photoreceptor membrane, i.e. rhodopsin and the lipid bilayer. Therefore, we have compared properties of rhodopsin in native rod outer segment membranes with those of rhodopsin in an experimentally changed micro-environment. The alterations are achieved by phospholipase C treatment of photoreceptor membranes, which results in a reduction of the amount of amphipathic lipids, by complete removal of all lipids by means of affinity chromatography, which results in suspensions of pure rhodopsin, and by reconstitution of membranes of selected lipids and rhodopsin. The procedures to obtain the various preparations are described in chapter 3, together with materials and methods used throughout this study.

Since rhodopsin probably is related to ion fluxes across the photoreceptor membrane, it is important to know to which extent rhodopsin is incorporated in the membrane and whether (which) functional parts of the visual pigment molecule are exposed to the aqueous phase or embedded in the hydrophobic core of the lipid bilayer. Therefore, the topography of the photoreceptor membrane is investigated using proteolytic enzymes (chapter 4). In order to see whether the lipid environment shields the protein molecule, the susceptibility of rhodopsin in the native membrane towards proteases is compared to that of rhodopsin in detergent solution, in its lipid- and detergent-free, pure state and in phospholipase C treated membranes.

Subsequently, the effects of the above-mentioned modifications of the micro-environment of rhodopsin are studied by means of the parameters described in section 2.6. However, in bovine rod outer segment membrane suspensions the photolytic sequence of rhodopsin is ill-defined. In order to be able to use the late part of this sequence as a parameter, the decay products of metarhodopsin II are spectrophotometrically and chemically characterized in native photoreceptor membrane suspensions (chapter 5). The effects of environmental changes on the metarhodopsin II appearance and decay are subsequently compared with the behaviour in native membranes (chapter 6). The results of the determination of the thermal stability and the regeneration capacity of the various preparations are presented in chapter 7. Electronmicroscopic studies of photoreceptor membranes treated with phospholipase C are presented in chapter 8.

In the final chapter (chapter 9) the results of the preceding chapter are interpreted in the context of rhodopsin-lipid relationships. Tentative conclusions about the location of rhodopsin in the lipid bilayer and about the role of lipids in the maintenance of the structural and functional integrity of rhodopsin are presented. Some proposals for further investigations along these lines are made.

CHAPTER 3

MATERIALS AND GENERAL METHODS

3.1. Introductory remarks

In this chapter a description is given of materials, procedures and determinations, which have been used throughout this work. Results which have been obtained to verify the accuracy and correctness of the procedures are also included in this chapter. Methods which are restricted to detailed parts of the work are presented in the respective chapters.

Whenever rhodopsin preparations are involved, all manipulations are carried out in darkness or under dim red light (λ > 650 nm), unless indicated otherwise.

3.2. Materials

The specific reagents are listed together with the suppliers.

Concanavalin A-sepharose 4B: Pharmacia, Uppsala, Sweden.
Triton X-100: British Drug Houses Ltd, Poole, England.
Digitonin and papain (EC 3.4.4.10): Merck, Darmstadt, W. Germany.
Cetyltrimethylammonium bromide (technical grade); dodecyltrimethylammonium bromide (recrystallized from ethanol/ethylacetate before use) and all-trans retinaldehyde: Eastman-Kodak, Rochester, N.J., U.S.A.
Emulphogene BC-720: General Anilin and Film Corp., Delft, The Netherlands.
NADPH; pepsin and cytochrome-c: Boehringer, Mannheim, W. Germany.

Pronase (a mixture of proteases from Streptomyces griseus) and Coomassie blue R250: Serva, Heidelberg, W. Germany.

α-Chymotrypsin (EC 3.4.4.5, bovine pancreas); α-chymotrypsinogen, β-lactoglobulin; subtilisin (EC 3.4.4.16, subtilopeptidase A); ovalbumin and myoglobin: Sigma, St. Louis, Mo, U.S.A.

Trypsin (EC 3.4.4.4.): Worthington, Freehold, N.J., U.S.A.

Bovine serum albumin (BSA): Behringwerke AG, Marburg, W. Germany.

α-Methylmannose (α-methylmannopyranoside): Calbiochem, San Diego, Ca, U.S.A.

$NaCNBH_3$ (recrystallyzed according to Borch et al., 1971): Aldrich-Europe, Beerse, Belgium.

$NaBH_4$: Fluka, Switzerland.

Mono-, di-, tri-oleoylglycerides; didecanoyl- and dioleoyl-phosphatidylcholine. Serdary Research Lab. Inc., London, Ontario, Canada.

Phosphatidylcholine (egg); phosphatidylethanolamine (egg) and phosphatidylserine (bovine spinal cord), all of purest grade: Lipid Products, South Nutfield, England.

Monogalactosyldiglyceride from Bifidobacterium Bifidum (Veerkamp, 1972) has been kindly supplied by Dr. J.H. Veerkamp.

Phosphatidic acid is obtained from phospholipase D treated egg-phosphatidylcholine. Purity of lipids is checked by thin layer chromatography.

Lipids of rod outer segment membranes are isolated by extraction with chloroform-methanol.

Phospholipase C (Bacillus cereus, EC. 3.1.4.3) is purified essentially following the procedure of Otnaess et al. (1972).

The 11-cis retinaldehyde is isolated following the procedure of Brown and Wald (1956), as modified by Rotmans (1973).

All other reagents are obtained commercially and of the highest purity available.

3.3. General methods

3.3.1. Isolation of rod outer segment membranes

The rod outer segment membranes are isolated from bovine eyes. The eyes are obtained from the local slaughterhouse, where they are excised within 10 minutes after the death of the animal and placed in a light-tight container at room temperature. Upon arrival in the laboratory the retinas are dissected within three hours post mortem, and are immediately immersed in ice cold 0.16 M Tris-HCl buffer (pH 7.2). All manipulations are carried out in darkness or in dim red light (λ > 650 nm).

The rod outer segments are isolated by means of continuous sucrose gradient centrifugation according to the procedure of De Grip et al. (1972). The rod outer segments appear in a single band (density ~1.10), and are taken from the gradient by means of a pipet.

Intact rod outer segments are obtained by diluting this suspension dropwise with an equal volume of the above Tris-HCl buffer, while shaking mildly. Then the suspension is centrifuged during 30 min at 4°C (SS34 rotor, Sorvall RC2B, 5000 rpm), the pellet is resuspended in 0.16 M Tris-HCl (pH 7.2), containing 13% sucrose, and centrifuged under the same conditions as before. This procedure is repeated once more and the resulting pellet is resuspended in the sucrose-Tris-HCl buffer. Rod outer segments thus obtained are fairly intact as judged by observation through a phase-contrast microscope (fig. 3.1). Rod-like structures are clearly visible, although several rods are bent or broken. Electronmicroscopic observation reveals stacked discs (cf. fig. 1.3), partially surrounded by the plasma membrane.

Suspensions of photoreceptor membranes are obtained by washing the pellet of rod outer segments, resulting from the first sedimentation, twice with distilled water at 4°C (30 minutes, 20,000 rpm, SS34 rotor, Sorvall RC2B). The pellet is either lyophilized after washing with distilled water, or resuspended in an appropriate volume of the desired buffer. When the suspensions are not immediately used

Fig. 3.1. Phase-contrast micrograph of isolated bovine rod outer segments, washed and resuspended om 0.1 M Tris-HCl (pH 7.2) containing 13% sucrose.

they are stored in a light-tight box at -70°C, after quick freezing (solid CO_2/ethanol). Suspensions thus obtained contain lysed rod outer segments and can be described as photoreceptor membrane suspensions. Electronmicroscopic observation shows that after washing with distilled water membrane vesicles are obtained, which do no longer have the characteristic flat discal structure (fig. 4.3).

In the isolation procedure of De Grip et al. (1972) the rhodopsin content of the rod outer segment membranes is enriched by treatment with excess 11-cis retinaldehyde, which converts all opsin into rhodopsin. Excess retinaldehyde is then, after addition of NADPH, reduced to retinol by the retinoldehydrogenase present in photoreceptor membranes. The retinol can be removed in a second sucrose gradient. This enrichment step is used, when a preparation must be free of opsin. Care must be taken to remove all retinol, since this may interfere in some analytical determinations (see section 5.2.3). Seasonal effects on the amount of opsin in an un-

enriched preparation (De Grip et al., 1972) have been reduced by performing excision of the eyes at the slaughterhouse in a more light-shielded place and sooner after death of the animals. Now the unenriched photoreceptor membranes never contain more than 10% opsin, as judged by incubation with 11-cis retinaldehyde (see 3.3.4). For this reason the enrichment step and the second gradient centrifugation have usually been omitted when the presence of some opsin is of minor importance, e.g. in most spectral transition measurements.

The preparations obtained by these procedures show excellent spectral characteristics, with A_{280}/A_{500} spectral ratios of 2.0 to 2.3, measured in detergent, and an A_{400}/A_{500} ratio of 0.20-0.25. Rhodopsin is the dominant protein in these photoreceptor membrane preparations (cf. fig. 4.2.A), and accounts for at least 85% of all protein present (De Grip et al., 1972; Daemen et al., 1972). The phospholipid/rhodopsin molar ratio is found to be 60 to 70.

3.3.2. Spectral measurements

A Zeiss PMQ II or a Rapid T3 spectrophotometer (Howaldtswerke-Deutsche Werft, Kiel, W. Germany) are used for measurements of whole spectra or at a single wavelength. Both instruments are located in a dark room and all instrumental light is excluded, so that preparations can be inserted without the risk of undesired illumination.

The Rapid T3 is a double beam spectrophotometer and is adapted for spectral measurements of turbid solutions (suspensions), since the thermostated cuvettes are placed immediately in front of an integrating sphere photomultiplier system. Spectra are recorded down from 600 nm at a scanning speed of 4 or 6 nm/second. The intensity of the detection beam is low enough that continuous measurement of rhodopsin preparations at 500 nm causes no detectable loss of rhodopsin within 1 hour.

3.3.3. Determination of rhodopsin content

The rhodopsin concentration is spectrally determined from the difference in 500 nm absorbance before and after complete illumina-

tion (ΔA_{500}) in a detergent solution (digitonin, Triton X-100, Emulphogene BC-720, sodiumcholate, octylglucose, cetyl- or dodecyl-trimethylammonium bromide). Lyophilized preparations are solubilized in a specific volume of a buffered detergent solution (1% w/v detergent), or detergent is added to a rhodopsin containing suspension to a final concentration of 1%. To exclude interference from photoproducts at 500 nm, NH_2OH (1 M in bicarbonate, pH 6.5) is added to a final concentration of 50 mM. Hydroxylamine converts all-trans retinaldehyde quickly and completely into retinylideneoxime (λ_{max} = 365 nm), which does not absorb at 500 nm. Rhodopsin itself is resistant against NH_2OH. Therefore, the absorbance difference at 500 nm before and after illumination can be completely attributed to rhodopsin. The results are expressed for suspensions in ΔA_{500}/ml and for lyophilyzed material in ΔA_{500} per mg dry weight (ΔA_{500}/mg). From the absorbance difference at 500 nm and a molar absorbance of 40,500 (Wald and Brown, 1953; Daemen et al., 1972), the molar rhodopsin concentration or content can be calculated.

The buffer in which rhodopsin preparations are suspended or solubilized by detergent is of minor importance, since neither pH (4 to 8), nor ionic strength (0 to 1 M), nor the type of buffer salts used have any effect on the 500 nm absorbance of rhodopsin (between -70°C and 30°C). Generally 0.1 M phosphate (pH 6.5) or 0.1 M Tris-HCl (pH 7.3) is used. Some detergents have deleterious effects on rhodopsin at elevated temperatures (e.g. dodecyltrimethylammonium bromide) and measurements must be carried out at 0-4°C (see section 3.3.7).

Exhaustive illumination of all rhodopsin present is achieved by placing the preparation behind 3 mm thick KG1 (infrared) and OG_{530} (cut-off wavelength 510 nm) filters (Scott-Jena, Mainz, W. Germany), in front of a 300 W tungsten lamp at a distance of 50 cm for 10 minutes.

3.3.4. Regeneration capacity

The regeneration capacity of a rhodopsin preparation is the percentage of the original rhodopsin molecules that, after illumination, can be reconverted into rhodopsin, by treatment with 11-cis retinaldehyde. It is determined in 0.1 M phosphate buffer (pH 6.5) at room temperature. A sample of a suspension or detergent solution, is taken to estimate the rhodopsin concentration before illumination (A). A second sample is exhaustively illuminated (see section 3.3.3) and in one part the residual rhodopsin concentration (B) is determined. To another part a five-fold molar excess of 11-cis retinaldehyde on the basis of initial rhodopsin content is added. The 11-cis retinaldehyde is solubilized in methanol, so that the final methanol concentration in the preparation never exceeds 3%. The resulting mixture is incubated at room temperature for 1.5 hr, and the rhodopsin concentration is measured (C). The regeneration capacity is calculated as (C-B)/(A-B) x 100%. For preparations that initially contained opsin a value exceeding 100% may be found. In that case a non-illuminated sample is also incubated with 11-cis retinaldehyde and the rhodopsin concentration is determined (D). The regeneration capacity is then calculated by the formula (C-B)/(D-B) x 100%, while the initial percentage of opsin is calculated by (D-A)/D x 100%.

To suspensions, which are unstable and show spontaneous sedimentation, glycerol is added to a final concentration of 25% to keep the preparation homogeneous. Glycerol does not influence the regeneration capacity.

3.3.5. Thermal stability

The thermal stability of a rhodopsin preparation is determined in 0.1 M phosphate buffer (pH 6.5), either in the presence or absence of detergent. An aliquot of 200 µl of the preparation (about 30 nmol rhodopsin/ml) is inserted into a test-tube, which is preheated in a thermostated waterbath. After a certain incubation

period, the test-tube is taken out and rapidly cooled in a mixture of ethylalcohol and solid CO_2. The ΔA_{500} is measured at about 4°C after addition of ice-cold Triton X-100 and hydroxylamine to final concentrations of 1% (w/v) and 50 mM, respectively. At each temperature at least five samples are incubated for different periods of time.

The decay of the 500 nm absorbance at a certain temperature consistently obeys first order kinetics. By plotting the relative amount of rhodopsin remianing after 10 minutes of incubation at different temperatures against those temperatures, the temperature at which 50% of thodopsin is denatured in 10 minutes is found by interpolation. This value in °C is used as a relative measure for the thermal stability.

3.3.6. Determination of phosphate and lipid/rhodopsin molar ratio

The amount of phospholipids in a preparation or the amount of phosphate esters, liberated during phospholipase C treatment of membranes, are determined as inorganic phosphate after acid destruction, using KH_2PO_4 as a standard (Broekhuyse, 1968).

When large quantities of sugars are present the sample is heated with 30% H_2O_2 at 90-100°C before acid destruction in order to prevent carbonization.

Since the phospholipids are known to contain only one phosphate residue per molecule, the lipid/rhodopsin molar ratio can be calculated from the amounts of lipid-phosphate and rhodopsin in a particular preparation. Phosphate determination of either water washed photoreceptor membranes or of their chloroform-methanol extracts yields identical lipid/rhodopsin molar ratios, indicating that contributions of protein-bound phosphate are negligible.

3.3.7. Purification of rhodopsin by means of affinity chromatography

The procedure is based on the fact, that concanavalin A has a high affinity for the sugar residues attached to rhodopsin (Steinemann and Stryer, 1973). A concanavalin A-sepharose 4B column

(10 cm long, 1 cm diameter) is packed and equilibrated (and, if necessary, stored) in buffer A (0.1 M acetate pH 6.0, 1 M NaCl, 1 mM dithioerythritol, 1 mM $MgCl_2$, 1mM $MnCl_2$, 1 mM $CaCl_2$ and 0.1 M of the detergent dodecyltrimethylammoniumbromide (DTAB)). Fresh rod outer segment membranes from 45 bovine eyes are isolated by means of the procedure described in section 3.3.1. The suspensions are twice washed with water, solubilized in buffer B (buffer A containing 0.1 M instead of 1 M NaCl), and centrifuged at 15,000 x g for 20 minutes at 4°C. The clear supernatant contains 60 nmol rhodopsin/ml and 9 ml of this solution is applied to the column by means of a peristaltic pump. Fractions of 3 ml are collected at a constant flow rate of 6 ml/hr. A volume of buffer B equal to at least four times the column volume is eluted (usually overnight), before rhodopsin is driven from the column by buffer C, consisting of buffer B to which 0.2 M α-methylmannose (α-methylmannopyranoside) is added. All manipulations are carried out at 0-4°C and in darkness or dim red light.

Absorbances at 278 and 500 nm of the fractions are measured at 4°C after suitable dilution with appropriate buffer. Since the elution of rhodopsin coincides completely with the appearance of α-methylmannose, corrections for the absorbance of the buffers at 278 nm must be made. The concentration of α-methylmannose is determined in each fraction by optical rotation measurements. The contribution of rhodopsin to the optical rotation appears to be negligible. The phospholipid content of the fractions is measured as described in section 3.3.6. When not immediately used, the fractions are quickly frozen and stored at -70°C.

The elution pattern resulting from this procedure is shown in figure 3.2. The fractions containing phospholipids are completely separated from those containing rhodopsin. The recovery of the total 500 nm absorbance and of the total amount of phospholipids is invariably better than 90%. Acid destruction of the fractions with the highest rhodopsin concentration does not yield any detectable inorganic phosphate. This implies that less than 0.3 mol phospholipid per mol rhodopsin remains, which is less than 0.5% of

the original amount of phospholipids.

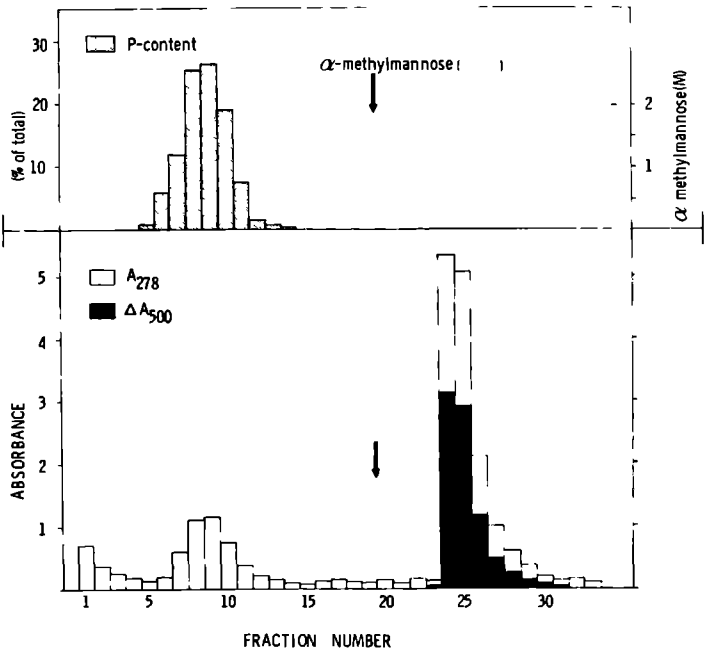


Fig. 3.2. Affinity chromatography over a concanavalin A-sepharose 4B column of rod outer segment membranes solubilized in 0.1 M dodecyltrimethylammonium bromide (see text). The procedure takes place at 0-4°C in darkness. The column has a length of 10 cm and a diameter of 1 cm. The flow rate is 6 ml/hr and 3 ml fractions are collected. The total amount of rhodopsin applied is 68 nmol/ml gel at a concentration of 60 nmol/ml buffer B.

The highest rhodopsin concentration coincides with the front of the α-methylmannose elution (fig. 3.2). Starting from solutions, which contain 60-100 nmol rhodopsin per ml, this concentration increases up to 190 nmol/ml of buffer C for a 10 cm column, and up to 440 nmol/ml for a 40 cm column. These high concentrations are only reached when the amount of rhodopsin applied approaches the maximal binding capacity of the column. When less material is applied to the column, rhodopsin is distributed over a greater volume during elution. This is probably due to retardation as a result of unoccupied concanavalin A molecules in the lower part of the column.

The concentration of the rod outer segment membrane solution applied to the column does not appear to be critical, provided that care is taken to elute a volume of buffer B large enough to wash all phospholipids from the column.

When the amount of rhodopsin applied exceeds the binding capacity of the concanavalin A present, the excess of rhodopsin will automatically be eluted, though slightly retarded as compared to the elution of the phodpholipids. Under these conditions the column becomes maximally loaded and a maximal binding capacity of about 110 nmol rhodopsin/ml sedimented gel can be calculated from the volume of the column packing and the total amount of rhodopsin that remains bound.

The elution of rhodopsin is most effective when 0.1-0.2 M α-methylmannose is used. With 0.1 M D-glucose, as used by Steinemann and Stryer (1973), elution is less satisfactory, since rhodopsin is then recovered in at least 10 times lower concentrations than with α-methylmannose. This is not surprising, since concanavalin A has a much higher affinity towards α-acetals of a carbohydrate (cf. Goldstein et al., 1965; Sharon and Lis, 1972).

The column can be used at least five times over a period of 6 weeks, without any detectable loss in binding capacity, provided that it is stored in buffer A at $4^{\circ}C$.

Figure 3.3 shows the absorbance spectrum of a purified rhodopsin preparation. It is relatively independent of the quality of the starting material. Rod outer segment membrane preparations with A_{278}/A_{500} ratios of up to 3.9 can be completely purified (table 3.1).

The thermal stability (see section 3.3.5) of rhodopsin in dodecyltrimethylammonium bromide solution decreases after removal of phospholipids. The temperature at which 50% of the 500 nm absorbance is lost in 10 min is $38.7^{\circ}C$ for rod outer segment membranes solubilized in 100 mM dodecyltrimethylammonium bromide solution (pH 6.5), whereas it is decreased to $24.0^{\circ}C$ after purification. Nevertheless, lipid-free rhodopsin can be stored at $0-4^{\circ}C$ for at least 24 hr without detectable loss of 500 nm absorbance.

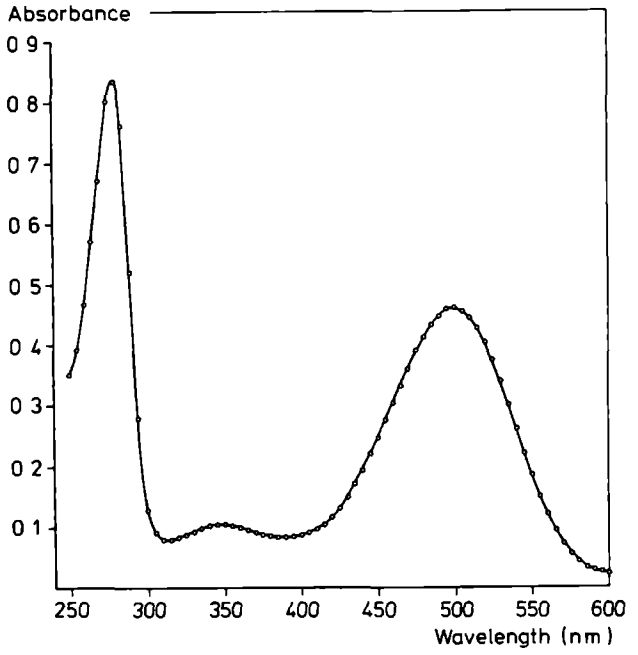


Fig. 3.3. Absorbance spectrum of purified rhodopsin in buffer C (see text), corrected for the absorbance contribution of the buffer. The dilution is arbitrarily chosen.

Table 3.1

CHARACTERISTICS OF PHOTORECEPTOR MEMBRANES AND PURIFIED RHODOPSIN SOLUBILIZED IN DODECYLTRIMETHYLAMMONIUM BROMIDE*

	A_{400}/A_{500}	A_{278}/A_{500}	mol phospholipid per mol rhodopsin
photoreceptor membranes	0.20-0.25	2.3-3.9	60-70
purified rhodopsin	0.18-0.23	1.6-1.8	< 0.3

*DTAB concentration 100 mM at 4°C and pH 6.

Samples before and after affinity chromatography have been subjected to sodium dodecylsulfate (SDS) gel electrophoresis according to Weber and Osborn (1969) with 10.6% gels. Protein bands are

stained according to Fairbanks et al. (1971). Prior to electrophoresis DTAB is removed by dialysis (see section 3.3.8) from the sample in order to prevent precipitation upon addition of SDS. The resulting rhodopsin preparation is solubilized in 0.01 M phosphate (pH 7.0) containing 2% SDS and 1% β-mercaptoethanol, and is then subjected to electrophoresis. Figure 3.4 shows the result of puri-

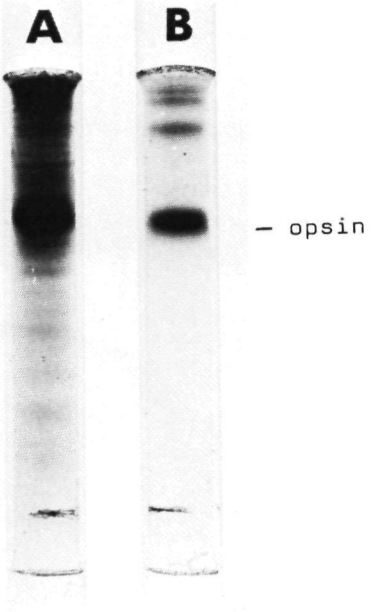


Fig. 3.4. Sodiumdodecylsulfate polyacrylamide gel electrophoresis of rhodopsin preparations. A. before purification; B. after purification over a concanavalin A-sepharose 4B column. Dodecyltrimethylammonium bromide is removed by dialysis, prior to electrophoresis according to Weber and Osborn (1969) with 10.6% gels. Protein bands are stained with Coomassie blue R-250 (Fairbanks et al., 1971).

fication of a purposely impure rod outer segment preparation. The protein impurities have disappeared, but in addition to the normal opsin band (MW 36,500) some bands with higher molecular weights are present. They probably represent oligomeric forms of opsin, since bands with the same mobility are obtained from pure rod outer segment preparations in the absence of reductive agents (cf. De Grip et al., 1975a). When the normal, purer rod outer segment membranes are subjected to affinity chromatography, the oligomeric forms are much less pronounced or completely absent.

No concanavalin A is detected in the eluted fluid by gelelectro-

phoresis. Glycoproteins other than rhodopsin and also glycolipids possibly would not be separated from rhodopsin by the present method. But if they are present at all, their concentration must be very low, since they have not been found by lipid and sugar analysis (cf. De Grip, 1974) and staining for sugars after SDS gel electrophoresis does not reveal detectable amounts of glyco-compounds other than rhodopsin (compare fig. 4.2.A).

Opsin is probably separated from rhodopsin in this purification procedure (De Grip, personal communication). Nevertheless, any risk of opsin contamination can always be avoided by its conversion into rhodopsin with 11-cis retinaldehyde during the isolation of the photoreceptor membranes (section 3.3.1). This conversion is impossible after the purification procedure, since if opsin has been in contact with DTAB, it completely fails to react with 11-cis retinaldehyde to form rhodopsin.

The purified rhodopsin solutions thus obtained are very suitable for membrane reconstitution procedures, since they are lipid-free and the detergent DTAB can be removed by dialysis (section 3.3.8).

The affinity chromatography procedure offers some clear advantages above purification by means of hydroxylapatite columns (Hong and Hubbell, 1972, 1973; Applebury et al., 1974). It results in rhodopsin solutions in phosphate-free buffers, so that no exhaustive dialysis is required before the residual amount of phospholipids can be determined. Furthermore, rhodopsin concentrations at least 5 times higher than those obtained after purification with hydroxylapatite are obtained, so that much less dialysis buffer is needed to remove the detergent. Finally, large quantities of rhodopsin can be purified with relatively small columns.

3.3.8. Preparation of pure rhodopsin and reconstitution of rhodopsin into lipid bilayers

The isolation of pure, lipid- and detergent-free rhodopsin is achieved by dialysis of pooled rhodopsin containing fractions, ob-

tained after affinity chromatographic purification. The dialysis procedure is identical to the one described below for membrane reconstitution. The pellets of pure rhodopsin, obtained after dialysis and centrifugation, are much more transparent than those of rod outer segment membrane preparations or reconstituted membranes and they have a gel-like appearance (cf. Applebury et al., 1974). Pure rhodopsin is resuspended in 0.1 M phosphate buffer (pH 6.5) or for reconstitution experiments in 0.1 M acetate buffer (pH 6.0) to avoid interference in the determination of phospholipid/rhodopsin molar ratio.

The reconstitution of lipid bilayers containing rhodopsin is performed essentially according to a procedure described by Hong and Hubbell (1972, 1973). Three different rhodopsin preparations are used for reassembly with lipids in order to investigate the properties of rhodopsin in the resulting vesicles of reconstituted membranes (see chapter 6, 7 and 8). These preparations are (1) solutions of lipid-free pure rhodopsin, obtained by affinity chromatography in buffered 100 mM DTAB (buffer C, see section 3.3.7), or (2) pure rhodopsin suspensions in 0.1 M acetate buffer (pH 6.0) isolated as described in the previous paragraph, or (3) phospholipase C treated rod outer segment membranes suspended in 0.1 M acetate buffer (pH 6.0). The reconstitution procedure is similar for the three preparations. The desired type and amount of lipids, dissolved in chloroform, is dried in a test-tube by a stream of nitrogen. Then, at 0-4^{o}C in dim red light, a concentrated rhodopsin preparation (about 80 nmol/ml) and solid dodecyltrimethylammonium bromide to a final concentration of 300 mM are added. A glass-pearl is inserted to facilitate lipid solubilization. The test-tube is shaken under N_2 atmosphere until a clear solution is obtained, which generally takes about one hour. Subsequently the solution is transferred to a pre-cooled dialysis bag and dialyzed against pre-cooled HEPES-buffer (5 mM, pH 6.6) with 1 mM EDTA and 1 mM DTE, using 250-500 ml buffer per ml rhodopsin solution. The dialysis buffer is stirred continuously under a nitrogen atmosphere in a cold room (4-6^{o}C), while the vessels are placed in an ice-bath to

avoid heating by the magnetic stirrer. Dialysis is continued for 72 to 124 hrs and the buffer is refreshed at least four times.

The reconstituted membranes precipitate in the dialysis bag and they are harvested by centrifugation at 75,000 x g (1 hr, 4°C). A small amount is used to determine the lipid/rhodopsin molar ratio (3.3.6). The remainder is suspended in 0.1 M phosphate buffer (pH 6.5) and if necessary, stored at -70°C. Rhodopsin recoveries of 80-100% are obtained.

The ease by which homogeneity of the suspensions is obtained partially depends on the nature of the lipids. Reconstitution with phosphatidylserine gives a homogeneous and stable suspension by mere shaking, while with phosphatidylcholine sonication is required for proper dispersion. In the latter case addition of 10 to 30% glycerol is often required to avoid spontaneous sedimentation. Later experiments showed that the use of dialysis buffer with higher ionic strength, 0.1 M acetate (pH 6.0) or 0.1 M phosphate (pH 6.5) instead of HEPES, largely eliminates these difficulties, suggesting that increased ionic strength prevents the formation of compact membrane aggregates. Therefore, in later reconstitution procedures buffers of 0.1 M have occasionally been used, especially when phosphatidylcholine was involved.

Reconstitution of opsin into lipid bilayers is performed in similar fashion. Opsin is obtained by complete illumination of rhodopsin in DTAB solution. In all cases, the retinaldehyde liberated by illumination is not recovered in the opsin containing membranes, indicating that it is dialyzed out.

The recovery of total lipid in the reconstitution procedure is found to be at least 70%. Apparently these relatively small molecules do not easily pass the dialysis membrane, probably due to their initial incorporation into (mixed) micelles of large size.

3.3.9. Lipid addition

Mixing of rhodopsin preparations with lipids in the absence of detergent is performed by drying the desired amount of lipids

by a stream of nitrogen in a test-tube and subsequent addition of a suspension of pure rhodopsin or of phospholipase C treated membranes in 0.1 M phosphate buffer (pH 6.5). A glass-pearl is inserted and the mixture is vigourously shaken under N_2 atmosphere during 1 hr at room temperature in darkness. To improve homogeneity, the suspension is sonicated (3 times 2 seconds at 0°C) and the preparation is left at room temperature during at least one additional hour before further experimentation.

3.3.10. Phospholipase C treatment

Pure phospholipase C (EC 3.1.4.3) is isolated from Bacillus cereus, essentially according to the method of Otnaess et al. (1972). It is free of proteolytic activity. The lipolytic enzyme hydrolyses glycerophospholipids into water soluble phosphate esters and diglycerides (fig. 3.5). The activity of this zinc metalloenzyme

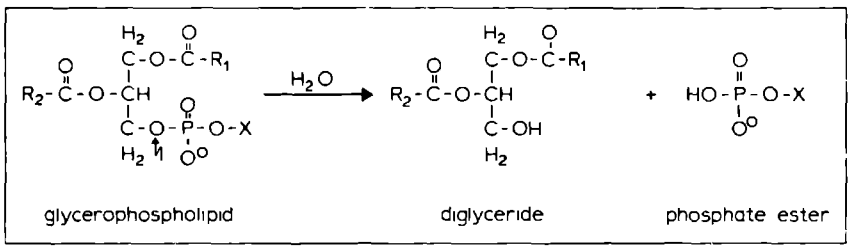


Fig. 3.5. Mode of action of phospholipase C on glycerophospholipids. R_1 and R_2 represent different hydrocarbon chains. X represents esterified polar alcohols, their structure is given in fig. 1.4.

is largely inhibited by EDTA or o-phenantroline (cf. Little and Otnaess, 1975). The phospholipase C preparation used in this study has a specific activity of 1950 units/mg, and is pure upon gel electrophoretic analysis.

Rod outer segment membrane suspensions are isolated as described in section 3.3.1, including washing with water. Alternatively the rod outer segment membrane suspension obtained from the sucrose gradient is diluted dropwise with an equal volume of 0.1 M Tris-HCl

(pH 7.2) and sedimented at 4°C for 30 min (3500 rpm, rotor SS34, Sorvall RC2B). The pellet is washed twice with 0.05 M Tris-maleate buffer (pH 7.0) under identical sedimentation conditions and finally resuspended in 0.1 M Tris-maleate (pH 7.0). The latter procedure results in more mildly lysed preparations than does the water washing procedure (compare fig. 4.3 and 8.4). The alternative procedure is used, since membrane structures of larger size are obtained, which enables the more easy interpretation of electron-microscopic details. Similar levels of phospholipid hydrolysis can be obtained in both types of preparations.

The phospholipase C treatment of rod outer segment membrane suspensions is performed in 0.1 M Tris-maleate buffer (pH 7.0) at room temperature under mild agitation in darkness. After incubation the enzyme activity is inhibited by the addition of 10 mM EDTA and cooling to 0°C. For electronmicroscopy the suspensions are sedimented at 4°C (30 min, 8000 rpm, rotor SS34, Sorvall RC2B) and the pellets are fixed or frozen as described in section 3.3.11 and 8.2.

The extent of phospholipid hydrolysis is calculated after acid destruction and phosphate determination (see section 3.3.6) of a sample of the homogeneous suspensions as well as of the supernatants (30 min, 20,000 rpm, Sorvall RC2B, rotor SS34). Since no phospholipids can be extracted from these supernatants, all phosphate measured must have resulted from liberated phosphate esters. The pellets are washed with water and resuspended in 0.1 M phosphate buffer (pH 6.5) for all further experimentation. Resuspension in 0.1 M acetate buffer (pH 6.0) is used for phospholipase C treated membranes with about 90% phospholipid hydrolysis, which are to be used for reconstitution (see section 3.3.8).

The rod outer segment preparations used have a phospholipid/rhodopsin molar ratio of 60 to 70. Incubation with sufficient enzyme leads to a maximal phospholipid hydrolysis of about 90% (cf. fig. 3.6) but invariably leaves rhodopsin spectrally completely intact. Different levels of phospholipid hydrolysis can be reached by varying the time of incubation or the phospholipase C concentration. Figure 3.6 presents experimental results of both procedures.

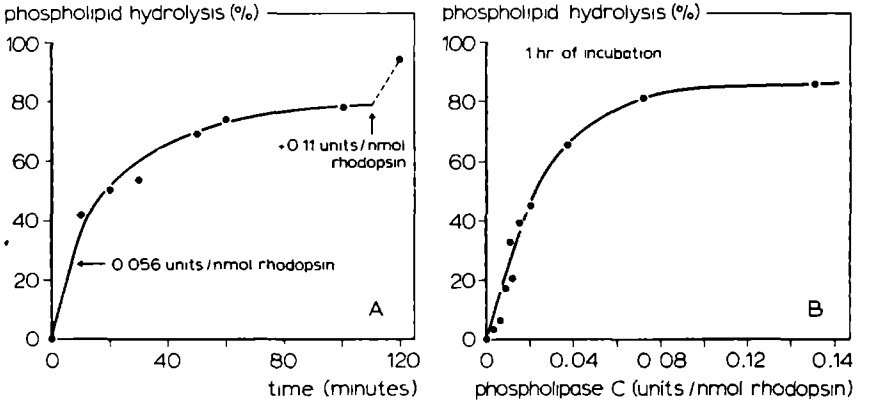


Fig. 3.6. Phospholipase C treatment of photoreceptor membrane suspensions. Incubation of 15 M rhodopsin at room temperature in 0.1 M Tris-maleate (pH 7.0). A: phospholipid hydrolysis in the presence of 0.056 units of phospholipase C per nmol rhodopsin. Extra phospholipase C is added after 110 minutes of incubation. B: phospholipid hydrolysis after 1 hour of incubation at varying phospholipase C concentration.

A curve as shown in fig. 3.6.A is not reproducible for different preparations and the pattern seems to depend on the extent of vesicle formation, the concentration of the membrane suspension and the phospholipase C concentration. The extent of phospholipid hydrolysis is more easily predicted upon variation of the phospholipase C concentration. Hence, this method is more commonly used in this study. At relatively low phospholipase C/rhodopsin ratios (roughly below 0.04 units/nmol rhodopsin) the addition of the enzyme leads to fast hydrolysis of phospholipids during the first 10 to 15 minutes, but further incubation hardly increases the level of hydrolysis. The same initial phase of the curve in fig. 3.6.B is thus obtained, when the incubation period is reduced to 15 minutes. The maximal level of hydrolysis is directly related to the amount of phospholipase C added as shown by the linearity of the initial phase of the curve in figure 3.6.B. This behaviour may be due to specific requirements in the structure of lipid bilayers

as substrate for phospholipase C as it seems to be the case for phospholipase C of Clostridium welchii (compare Dawson et al., 1976).

Phospholipase C of Bacillus cereus used in the present study is specific for glycerophospholipids, and prefers neutral glycerophospholipids (phosphatidylcholine and phosphatidylethanolamine) above negatively charged glycerophospholipids (e.g. phosphatidylserine or phosphatidylinositol). Table 3.2 illustrates the phospho-

Table 3.2

PHOSPHOLIPID COMPOSITION OF PHOTORECEPTOR MEMBRANES BEFORE AND AFTER PHOSPHOLIPASE C TREATMENT

PHOSPHOLIPID	NATIVE MEMBRANES % of total phospholipids*	PHOSPHOLIPASE C TREATED MEMBRANES % of original total phospholipids*
phosphatidylcholine	40.7	4.4
phosphatidylethanolamine	41.6	3.0
phosphatidylserine	15.5	7.2
phosphatidylinositol	2.0	2.0
TOTAL	99.8	16.6

*100% is equivalent to 65 mol phospholipid/mol rhodopsin.

lipid composition of a rod outer segment membrane preparation prior to and after phospholipase C treatment. The neutral phospholipids are more sensitive to phospholipase C treatment than the negatively charged phospholipids. Therefore, a preparation in which about 90% of the phospholipids are hydrolyzed contains phosphatidylserine as the major lipid component.

Phospholipase C treatment of mildly lysed rod outer segment membrane suspensions, where the discal membranes are unlikely to be everted, leads to a breakdown of phosphatidylcholine and phosphatidylethanolamine at identical rates and to the same extent. Since these two types of phospholipids comprise 70-80% of all photo-

receptor membrane phospholipids, this implies that conclusions about a possible asymmetry in the phospholipid distribution over both sides of the lipid bilayer in rod outer segment membranes cannot be derived from these experiments. Similar treatment of erythrocyte ghosts exhibits phospholipid asymmetry (cf. Verkleij et al., 1973; Zwaal et al., 1973, 1975; Gordesky, 1976). However, the cholesterol and sphingomyelin contents of erythrocyte membranes are much higher, while the level of phospholipid unsaturation is much lower than in the case of photoreceptor membranes. Moreover a structural support by a filamentous protein, like spectrin in the red cell membrane, is absent in rod outer segment membranes. In view of these differences in structure and composition, transfer of phospholipids from one side of the membrane to the other ("flip-flop") during phospholipase C treatment is more conceivable in pnotoreceptor than in erythrocyte membranes. Phospholipid asymmetry would then be much more difficult to detect (cf. section 9.5).

The addition of more than 0.07 units of phospholipase C per nmol of rhodopsin leads to a phospholipid hydrolysis of over 80% in 1 hour of incubation (cf. figure 3.6.B). A short additional incubation with a second amount of phospholipase C gives maximal hydrolysis (80-95%, cf. fig. 3.6.A) and is therefore frequently used. Since preparations with 80-90% hydrolysis exhibit identical characteristics, they will be referred to as preparations with "about 90% hydrolysis of phodpholipids".

The hydrolysis of phospholipids will inevitably cause changes in photoreceptor membrane organization, since amphipathic lipids are converted into neutral lipids. Therefore changes in the micro-environment of rhodopsin must occur. The implications of phospho-lipid hydrolysis by phospnolipase C for the photolytic behavior will be reported in chapter 6, while those for the thermal stability and regeneration capacity are presented in chapter 7. In chapter 8 the effects of the lipolytic treatment on the structure of the pho-toreceptor membrane will be discussed.

3.3.11. Electron microscopy

The electron microscopic studies reported in this thesis have been performed in co-operation with Prof. E.L. Benedetti and Dr. J. Olive of the Institut de Biologie Moléculaire, Université de Paris. The thin sections and replicas are made by Dr. J. Olive and Mr. M. Recouvreur. The micrographs are kindly supplied by Dr. J. Olive.

For thin sectioning concentrated suspensions of rod outer segment membranes are fixed in 2% glutaraldehyde in cacodylate buffer (pH 7.2) and then postfixed with 1% osmiumtetroxide. After dehydration by means of a series of ethanol solutions of increasing concentration, the samples are embedded in Vestopal W. Thin sections are stained with uranylacetate and leadcitrate and examined in a Philips 300 or 301 electronmicroscope.

The methods for freeze-fracturing are described in chapter 8 (section 8.2).

CHAPTER 4

ON THE TOPOGRAPHY OF PHOTORECEPTOR MEMBRANES

4.1. Introduction

Membranes in aqueous suspension spontaneously form closed structures (vesicles). If proteases are added to a suspension of membrane vesicles, the proteolytic action on membrane proteins will be governed by two restrictions. One is that the water-soluble enzyme molecules will not penetrate the membrane vesicles and will thus only act on the outside face of the membranes. The second is the extent of exposure of the membrane proteins to the aqueous phase in which the proteases are present. Proteins shielded by the hydrophobic core of the lipid bilayer may not or only to a limited extent become digested. For these reasons, proteases can be expected to be useful tools in elucidating the location of proteins in the lipid bilayer (topography of membrane proteins). Many successful applications have been reported in the literature (cf. e.g. Wallach, 1972; Brdiczka and Krebs, 1973; Wallach and Winzler, 1974; Steck, 1974).

At the time we started our investigations (1972) this approach had not been applied to photoreceptor membranes, although it was realized that rhodopsin is so strongly bound to the membrane, that it is only solubilized by detergent action. This suggested an intimate interaction of the protein with the membrane and probably a penetration into the lipid bilayer. The key role of rhodopsin in the visual process makes it important to know the location of this protein molecule with regard to the lipid bilayer. We have, therefore, studied the effects of proteases on intact and disturbed

photoreceptor membranes and on pure rhodopsin. Correlation of the degree of proteolysis of the visual pigment with the integrity of the photoreceptor membrane may give useful information on the protein-shielding capacity of the lipid bilayer and on the lipid--rhodopsin interactions.

The protease treatment of rhodopsin preparations has been performed both with specific proteolytic enzymes (trypsin and α-chymotrypsin) and with unspecific ones (papain, subtilisin, pronase). The most extensive studies have been carried out with pronase, a mixture of proteases from Streptomyces griseus, since this mixture has a very broad proteolytic action and should, therefore, have maximal effects on rhodopsin.

Much of the work described in this chapter has been published before (Daemen, Van Breugel and Bonting, 1974a and b; Van Breugel, Daemen and Bonting, 1975).

4.2. Methods

4.2.1. Enzyme treatment

Bovine rod outer segment membranes are isolated as described in section 3.3.1, including enrichment with 11-cis retinaldehyde to obtain opsin-free preparations. After washing twice with water the membranes are resuspended (1-3 mg/ml) in buffer and used within 16 hr after isolation. Incubations with pronase, subtilisin, α-chymotrypsin and trypsin are carried out in 0.1 M Tris-HCl, 5 mM $CaCl_2$ (pH 7.3) while in the case of papain $CaCl_2$ is replaced by 5 mM cystein and 2 mM EDTA. All incubations are performed at 30°C under mild agitation in the dark, unless otherwise indicated. The proteolytic enzyme concentrations are expressed in mg enzyme per 10 mg outer segment membranes (dry weight). Freshly prepared membranes have been used, since lyophilized preparations give poorly reproducible results upon enzyme incubation.

Solubilized amino-compounds are determined by the ninhydrin

method of Rosen (1957) in the supernatants (50,000 x g, 20 min) after enzyme inactivation and protein precipitation with 10% trichloroacetic acid (0°C). Leucine is used as a standard and results are expressed as leucine equivalents. Corrections for self-digestion of proteases are made.

4.2.2. Rhodopsin characteristics

The rhodopsin content either in suspension (ΔA_{500}/ml) or in lyophilized material (ΔA_{500}/mg) is determined as described in section 3.3.3. Inactivation of the proteolytic enzymes is not necessary when the determination of the rhodopsin content is carried out immediately after addition of detergent (usually Triton X-100 or Emulphogene BC-720). Alternatively, the decrease in 500 nm absorbance of a suspension is measured continuously by means of a Rapid T_3 spectrophotometer (see section 3.3.2).

The regeneration capacity and thermal stability are measured as described in sections 3.3.4 and 3.3.5.

Sulfhydryl groups are determined with Ellman's reagent according to the method of De Grip et al. (1973a).

4.2.3. Retinoldehydrogenase activity

Retinol dehydrogenase activity is determined by measuring the initial velocity of reduction of all-trans retinaldehyde at pH 6.5 and 37°C (Lion et al., 1975).

4.2.4. Gel electrophoresis

Membrane samples are washed twice with a solution containing 20 mM 1,4-dithioerythritol and 5 mM EDTA. Then ice-cold 10% trichloroacetic acid is added and after 30 min at 4°C the sediments are washed again. They are subsequently solubilized in a solution containing 1% dodecylsulfate and 1% β-mercaptoethanol in 0.01 M sodium phosphate buffer (pH 7.0) and dialyzed against this solution for 3 hr at room temperature. After another incubation for at least

1 hr at 37°C, glycerol is added to a final concentration of 5% and bromophenol blue is added as tracking dye. The resulting mixture is applied to the gel.

Dodecylsulfate disc gel electrophoresis is carried out according to Weber and Osborn (1969) with 10.6% gels or according to Laemmli (1970) with 13% gels, using an acrylamide/bisacrylamide weight ratio of 33. Both procedures give essentially the same results. Coomassie blue R250 staining is used for proteins and periodic acid-Schiff (PAS) staining for sugars (Fairbanks et al., 1971). Calibration proteins are: bovine serum albumin (MW 68,000), ovalbumin (MW 43,000), pepsin (MW 35,000), chymotrypsinogen (MW 25,700), myoglobin (MW 17,200) and cytochrome-c (MW 12,300). Electrophoretic analysis of membrane preparations after illumination in the presence of $NaBH_4$ is conducted with 0.2% propylgallate present in the upper running buffer (Hagins, 1973).

Prior treatment of the sample with trichloroacetic acid is necessary to inactivate proteolytic enzymes, which are not completely removed by washing with the 5 mM-EDTA-20 mM 1,4-dithioerythritol solution. Otherwise,the residual enzyme activity, which is greatly stimulated upon solubilization in dodecylsulfate buffer, leads to extensive degradation with formation of peptides with a MW of less than 6000 (determined by means of electrophoresis according to Swank and Munkres, 1971). Incubation of protease treated, trichloroacetic acid inactivated membranes with untreated outer segment membranes in dodecylsulfate and subsequent electrophoresis shows no additional digestion of the membrane proteins. The trichloroacetic acid treatment does not affect the electrophoretic pattern of the native membrane proteins.

4.3.1. Treatment of membrane suspensions with pronase

Incubation or rod outer segment membranes with pronase (0.1-2.0 mg/10 mg membranes) leads to release of water soluble amino compounds (fig. 4.1). Although the initial velocity of proteolysis

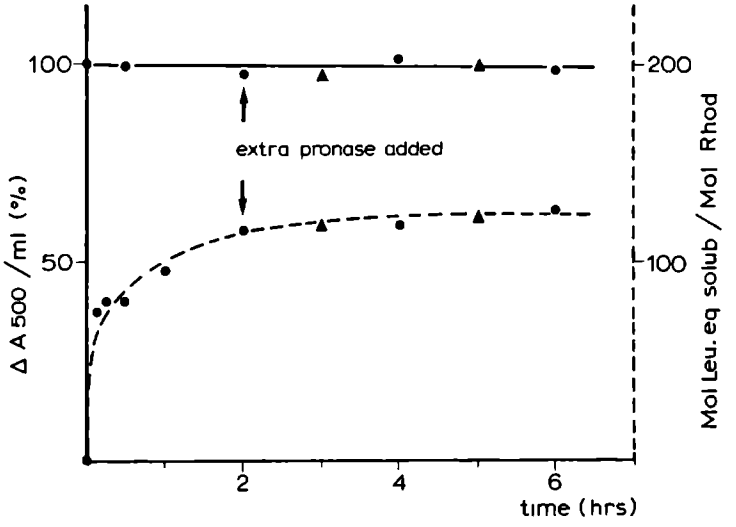


Fig. 4.1. Effect of pronase treatment of a rod outer segment membrane suspension. The membranes (about 2 mg/ml; 16 μM rhodopsin) are incubated with pronase (1 mg/10 mg membranes) in 0.1 M Tris-HCl, 5 mM $CaCl_2$ (pH 7.3) in darkness at 30°C. Differences in absorbance at 500 nm before and after illumination (●——●) are expressed as percentage of the value at time zero. Soluble leucine equivalents per mol rhodopsin (●---●) are corrected for contributions from the untreated suspension and from pronase. Closed triangles (▲) show both parameters after a second addition of an equal amount of pronase.

depends on the amount of pronase used, all pronase concentrations lead to approximately the same maximal level of digestion, while addition of fresh pronase at this point does not give rise to a further increase. The total amount of solubilized amino compounds varies somewhat with the preparation and amounts to 85 ± 25 leucine

equivalents per mol rhodopsin (n = 8). Incubation in darkness and in normal daylight give the same results.

During incubation in darkness the amount of rhodopsin, measured as ΔA_{500} before and after illumination, remains constant (fig. 4.1 and table 4.1). The regeneration capacity, determined by treatment

Table 4.1

PRONASE TREATMENT OF BOVINE ROD OUTER SEGMENT MEMBRANES

	No enzyme (%)	+Pronase (%)
ΔA_{500}/ml suspension	≡ 100	98 ± 2 (n = 10)
ΔA_{500}/mg membranes	≡ 100	108 ± 2 (n = 8)
Protein loss from ΔA_{500}/mg*	0	24
Phosphorus/mg membranes	≡ 100	113 ± 1 (n = 5)
Protein loss from P-determination*	0	31
Regeneration capacity	90	90
Retinol dehydrogenase activity	≡ 100	< 5

The preparations are incubated with 0.5-0.2 mg pronase per 10 mg membranes for 4 hr at 30°C in darkness. Standard deviations with the number of determinations (n) are included for some values.
*Based on an average protein content of rod outer segment membranes of 37% (De Grip et al., 1972).

of an illuminated suspension with 11-cis retinaldehyde, remains entirely unaffected. This is also the case for a preparation incubated with pronase in the light. The absorption spectrum of pronase treated rhodopsin is not affected by treatment with hydroxylamine (50 mM) or borohydride (2 mg/ml).

The thermal stability of rhodopsin is slightly decreased from 70.1°C in untreated to 66.7°C in treated membranes. The total number of SH groups, six per rhodopsin molecule (cf. De Grip et al., 1973a), is not affected by pronase treatment. Like in native preparations (cf. De Grip et al., 1975a) only two of the SH groups are readily accessible to Ellman's reagent in the absence of detergent. Finally, the characteristics of the spectral transitions after photolysis of

a pronase treated photoreceptor membrane suspension are identical to those of a native membrane suspension as reported in chapter 5.

In contrast, the retinol dehydrogenase activity of the membrane preparations is very rapidly abolished: after 15 min incubation less than 5% of the original activity remains, even at pronase concentrations of only 0.05 mg/10 mg membranes.

The amount of protein digested to water soluble compounds during enzyme incubation is determined by washing the pronase treated membranes three times with distilled water and then lyophilizing them. Absorption spectra, recorded for these preparations in detergent solutions (1% digitonin, Triton X-100, Emulphogene BC-720 or cetyltrimethylammonium bromide) do not differ from those of control preparations without pronase treatment, but the relative rhodopsin content (ΔA_{500}/mg) is increased by about 8% and the phosphorus content by 13% (table 4.1). Phosphorus containing compounds are not solubilized in measurable amounts during pronase incubation. From these data it is easy to calculate that between 24 and 31% of all membrane protein is digested to water-soluble products during pronase treatment (table 4.1).

SDS gel electrophoresis is used to study the effects of proteolytic treatment on the membrane proteins. Untreated outer segment membranes show the characteristic gel pattern with one major band of MW 36,500 $\pm$ 1500 and some minor bands with a MW of over 50,000 (fig. 4.2.A). Upon incubation with a small amount of pronase (0.1 mg/10 mg), the high molecular weight bands rapidly disappear. The major band becomes gradually weaker and is replaced by a band of MW 28,500 $\pm$ 1500 (fig. 4.2.B). The latter band derives from rhodopsin, since it contains an oligosaccharide residue characteristic for rhodopsin, as shown by sugar staining (PAS). It can also be shown that it contains the chromophoric binding site. The treatment with dodecylsulfate normally removes the chromophore. However, when the sample after incubation with pronase in darkness is illuminated in the presence of borohydride, which causes reductive fixation of the chromophoric aldimine bond, and then subjected to electrophoresis, the 28,000 band shows fluorescence upon illumination

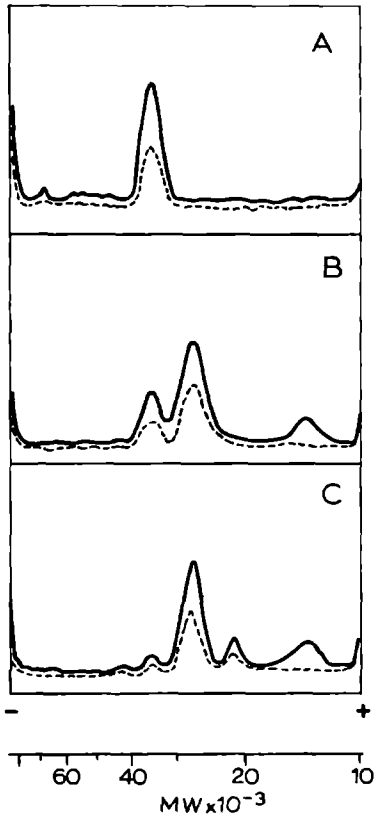


Fig. 4.2. Dodecylsulfate gel electrophoresis of rod outer segment membranes after pronase treatment. Weber and Osborn (1969) system with 10.6% gels. The experimental conditions are described in section 4.2. Scanning profiles at 550 nm after Coomassie blue staining for proteins (——) and at 520 nm after periodic-acid Schiff staining for sugars (---). A: native membranes; B: membranes after treatment with pronase (0.1 mg/10 mg membranes) for 15 minutes; C: membranes after pronase treatment (1 mg/10 mg membranes) for 15 minutes.

with 330 nm light. This fluorescence is also observed with N-retinyl opsin, formed from rhodopsin by reductive chromophore fixation with $NaBH_4$ during illumination (Bownds, 1967; Hagins, 1973).

Upon longer incubation or at higher pronase concentrations, an additional major band (MW 21,500 ± 1500) appears (fig. 4.2.C), which again carries an oligosaccharide and shows (weak) fluorescence upon electrophoresis after reductive fixation during illumination. The original rhodopsin band at 36,500 MW has now virtually disappeared, but complete digestion of the 28,500 band does not occur under these conditions. There also appears a broad and diffuse

band in the region of less than 15,000 MW (fig. 4.2.B and C), which does not contain oligosaccharide residues. It is not clear whether this band contains fluorescent protein upon reductive fixation during illumination, since free retinol may interfere in this region. Sometimes a low intensity band of MW 44,500 ± 1500 appears, which is most likely a dimer of the degradation product of rhodopsin, since it is PAS-positive. The gel pattern is the same, whether the pronase incubation is carried out in darkness (rhodopsin) or in normal daylight (opsin).

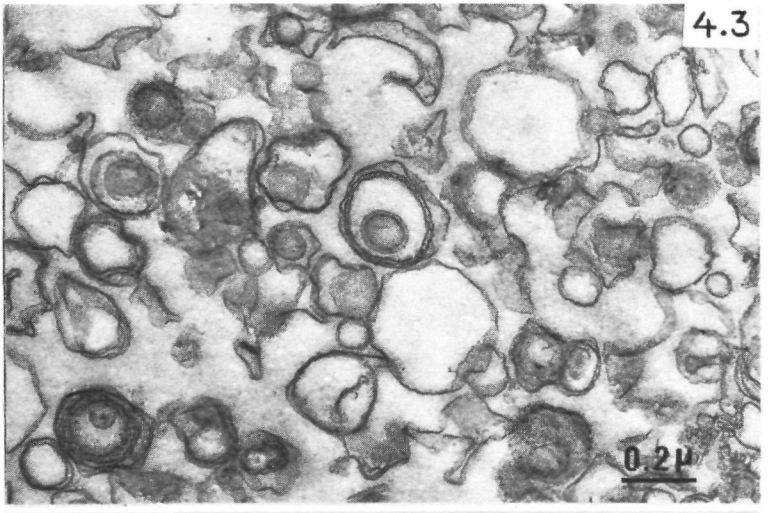
When intact rod outer segments are incubated with pronase, the degradation of rhodopsin is greatly inhibited as compared to lysed preparations under otherwise identical conditions, but the gel patterns are identical when rhodopsin is eventually attacked. This indicates that the plasma membrane acts as a permeability barrier for the proteolytic enzyme.

4.3.2. Electronmicroscopy

Thin section electronmicroscopy of the water-washed rod outer segment membrane suspensions shows that the preparation before incubation with pronase consists of predominantly single layered membrane vesicles of various size and shape (fig. 4.3). As may be clear from figure 4.4, this picture is not significantly altered, when incubation of the membrane suspension with pronase has resulted in fragmentation of all rhodopsin molecules, as judged by SDS gel electrophoresis (compare fig. 4.2.C). It is evident that pronase action does not lead to fragmentation or opening of the vesicles.

The only significant difference between untreated and treated photoreceptor membrane vesicles seems to be a more frequent direct contact between the membranes in the latter case (cf. fig. 4.4). This may indicate a reduction in membrane surface charge due to proteolysis.

Some preliminary results of freeze-fracturing of pronase treated membranes have been obtained (see fig. 8.10). Although the



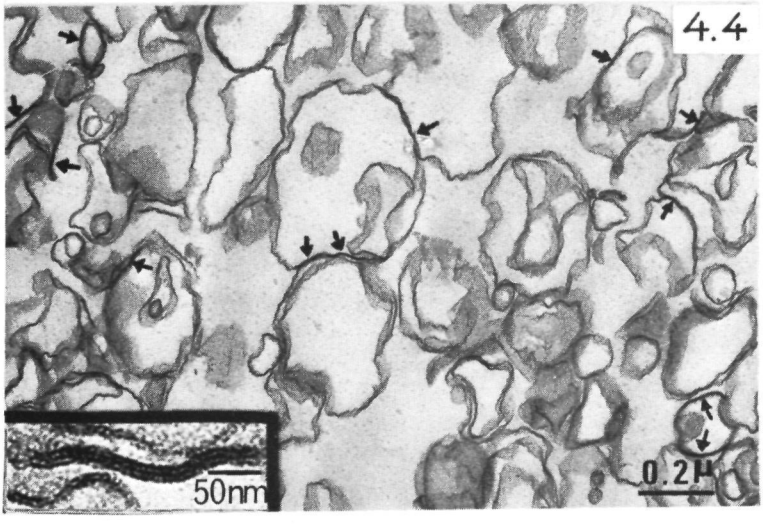


Fig. 4.3. Thin section electronmicrograph of rod outer segment membranes lysed by washing with water. For technical details see section 3.3.11.
Fig. 4.4. Thin section electronmicrograph of lysed rod outer segment membranes treated with pronase (0.5 mg/10 mg membranes) for 1 hr. The insert shows a higher magnification of an area of close intermembrane contact. These contacts are rather frequently found in these preparations (see small arrows).

pronase treatment seems to change the size and density of intramembranous particles at fracture-faces, the interpretation will have to await more extensive investigations.

4.3.3. Treatment of membrane suspensions with other proteases

Rod outer segment membranes have also been incubated with subtilisin, papain, α-chymotrypsin and trypsin. Over the entire range of enzyme concentrations (0.1-1.0 mg/10 mg membranes) the spectral integrity and regeneration capacity of rhodopsin remain unaffected, whereas the retinol dehydrogenase activity is again rapidly abolished by all four enzymes. None of the six SH groups per molecule of rhodopsin is removed by incubation with any of these enzymes and in membrane syspensions the accessibility of only two of the sulfhydryl-groups is also preserved. The photolytic behaviour of rhodopsin in the membranes remains completely unaffected by treatment with any of the proteolytic enzymes.

In the case of subtilisin, papain and α-chymotrypsin the occurrence of proteolysis is apparent from SDS-gel electrophoresis. The general digestion pattern is very similar to that with pronase, especially for subtilisin (fig. 4.5.A). However, upon papain treatment the 21,500 band does not appear (fig. 4.5.B), while with α-chymotrypsin the degradation, especially of the 28,500 band seems to proceed further (fig. 4.5.C). In contrast, incubation with trypsin (1 mg/10 mg membranes) has no effect on the rhodopsin band. With all four enzymes the high molecular weight bands (> 50,000) disappear as rapidly as with pronase.

4.3.4. Proteolytic treatment in detergent solution

In sharp contrast to all previous experiments, incubation of outer segment membranes in detergent solution does not show the limited susceptibility to proteolysis as seen in aqueous suspension. Addition of pronase to 1% detergent solutions of fresh rod outer segment membranes result in a relatively rapid decay of the spectral integrity of rhodopsin (fig. 4.6.A), as compared to pronase-free control experiments (fig. 4.6.B). The rate of the decrease in ΔA_{500}

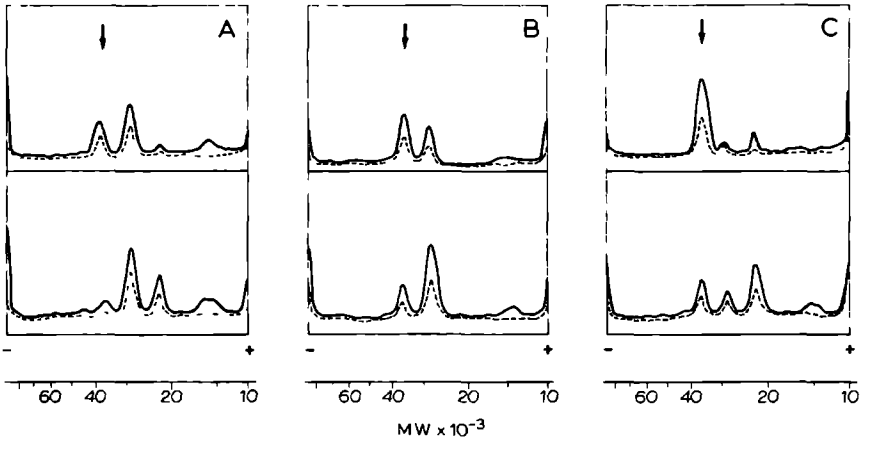


Fig. 4.5. Dodecylsulfate gel electrophoresis of rod outer segment membranes after treatment with proteases. Experimental conditions as in section 4.2. Scanning profiles at 550 nm after Coomassie Blue staining for protein (——) and 520 nm after periodic-acid Schiff staining for sugars (---). The arrow indicates the position of the opsin band. A: subtilisin; B: papain; C: α-chymotrypsin. Upper figures 0.1 mg protease/10 mg membranes, lower figures 1 mg/10 mg membranes. All incubations lasted 15 minutes.

follows the order of solubilizing power of the detergent used: digitonin < Triton X-100 < Emulphogene BC-720 < cetyltrimethylammonium bromide. This rate is linearly related to the pronase concentration in the range of 0.1-2.0 mg/10 mg membranes. At these pronase concentrations, and in all detergents used, the decay of rhodopsin obeys first order kinetics. At the same time a progressive protein digestion is observed as measured by the release of amino compounds. Up to 225 leucine equivalents/mol rhodopsin, as compared to 85 for suspensions, are found in 1% Triton X-100 solution under otherwise identical conditions.

The 500 nm absorbance of rod outer segment membranes solubilized in 1% detergent is more resistant against proteolytic attack by subtilisin, α-chymotrypsin, papain or trypsin than by pronase. At 37°C and concentrations of 2-5 mg enzyme per 10 mg membranes in detergent

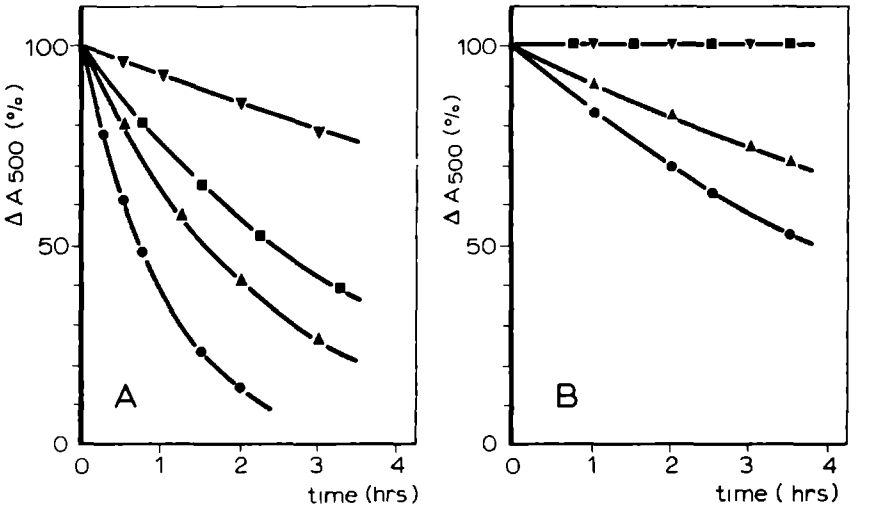


Fig. 4.6. Effect of pronase treatment on the 500 nm absorbance of rod outer segment membranes in 1% detergent solution. Incubation of the membranes (15 μM rhodopsin) in the presence (A) and absence (B) of pronase (1 mg/10 mg membranes) in 0.1 M Tris-HCl, 5 mM $CaCl_2$ (pH 7.3) in darkness at 30°C. Digitonin, ▼——▼; Triton X-100, ■—■; Emulphogene BC-720, ▲——▲; cetyltrimethylammoniumbromide, ●——●.

solution subtilisin, α-chymotrypsin, papain and trypsin cause decreases of 500 nm absorbance at rates that are similar to those already obtained at 30°C with 0.1-1 mg pronase per 10 mg membranes.

4.3.5. Proteolysis after phospholipid depletion

The experiments described in the previous paragraph suggest a relationship between membrane integrity and the susceptibility of rhodopsin to pronase. Therefore, we have also studied the effect of prior treatment of the membranes with phospholipase C. A preparation in which about 90% of the phospholipids are hydrolyzed into diglycerides and water-soluble phosphate esters (see 3.3.10), is subsequently treated with pronase. This leads to a moderately rapid decay of the spectral integrity of rhodopsin (fig.

4.7.A). The rate of decay again depends on the pronase concentration.

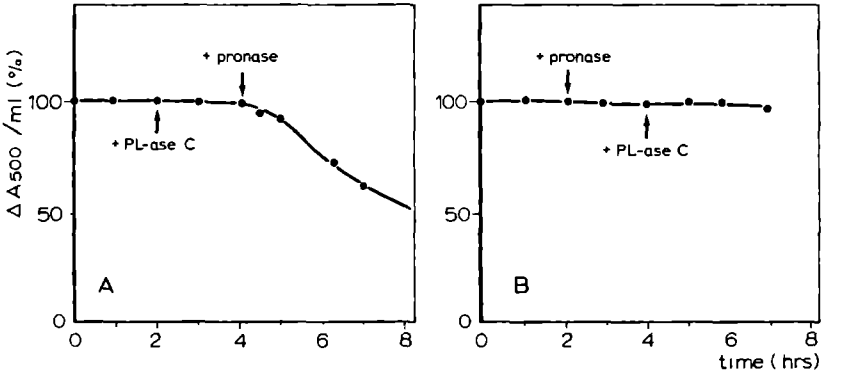


Fig. 4.7. Effect of pronase and phospholipase C on 500 nm absorbance of rod outer segment membrane suspension. The membranes are treated first with phospholipase C and then pronase (A) or in reversed order (B). The rhodopsin concentration is 20 μM (2.5 mg/ml photoreceptor membranes). Experimental conditions as in section 4.2, with 0.1 units of phospholipase C/nmol rhodopsin and 1.5 mg pronase/10 mg membranes. After preincubation for 2 hr the first enzyme is added, after another 2 hr the suspension is washed and the other enzyme is added.

An experiment, in which the incubations with lipolytic and proteolytic enzyme are reversed in order, shows that phospholipase C treatment in itself has no effect on the spectral integrity of pronase treated outer segment membranes (fig. 4.7.B). In this experiment the pronase is removed before the addition of phospholipase C, and the extent to which the membrane phospholipids are hydrolyzed amounts again to about 90%.

Pure lipid- and detergent-free rhodopsin (see 3.3.8) is also more susceptible to pronase action than rhodopsin in the intact membrane, and pronase treatment ultimately results in almost complete hydrolysis of rhodopsin. The rate of decrease of the 500 nm absorbance is even greater than in the case of phospholipase C treated membranes (see fig. 4.8), and is again dependent on the pronase concentration.

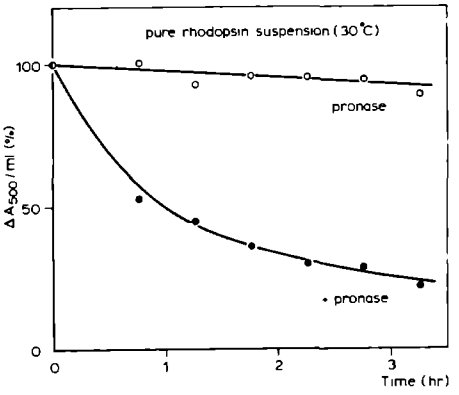


Fig. 4.8. Effect of pronase on 500 nm absorbance of pure, lipid- and detergent-free rhodopsin. The pure rhodopsin is incubated with (●——●) and without (o——o) pronase. Incubation of 20 μM rhodopsin in 0.1 M Tris-HCl (pH 7.3), in darkness, at 30°C. The pronase concentration is 1.5 mg/80 nmol rhodopsin, which is equivalent to the 1.5 mg pronase/10 mg membranes used in figure 4.7.

4.4. Discussion

4.4.1. Limited proteolysis of rhodopsin in native membrane environment

The most striking feature of protease treatment of rod outer segment membranes is the finding that rhodopsin is digested to a limited extent only. There is no loss of 500 nm absorbance or change in the absorption spectrum. Neither do the regeneration capacity and the photolytic behaviour show any effect of proteolysis. The thermal stability is only slightly affected and all six sulfhydryl groups remain present in the protease treated membranes without change in accessibility.

Nevertheless, it is evident from fig. 4.1 that proteolysis does occur. Between 24 and 31% of the total amount of membrane

protein is effectively removed from the membrane by treatment with pronase (table 4.1). This agrees fairly well with the value of 25% protein loss reported by Pober and Stryer (1975) for treatment with thermolysin. Since rhodopsin constitutes over 85% of all membrane protein (cf. fig. 4.2.A) a protein loss of over 24% implies that at least some part of rhodopsin must have been removed and degraded to small water-soluble fragments, even if all proteins other than rhodopsin have been degraded. This conclusion is supported by the observations of Saari (1974) and Virmaux et al. (1975), that thermolysin and papain do solubilize at least that part of rhodopsin which can be phosphorylated by endogenous protein kinase and ATP (Kuhn and Dreyer, 1972).

Electrophoretic analysis unambiguously shows that rhodopsin has indeed been attacked by the proteases. The first step in rhodopsin digestion seems to be very similar for all proteolytic enzymes, except for trypsin which does not affect rhodopsin. The first distinct digestion product appears to be a large fragment with a molecular weight of about 28,500. A fragment of about 8000 MW is probably split off in the first step, since no intermediate digestion products are found between the opsin band (36,500) and the 28,500 band.

The fate of the 8000 fragment may be twofold. Part of it is digested further to soluble fragments, contributing to the total protein loss of 24 to 31% (see second paragraph of this section). Other parts possibly remain in the membrane, because concomitant with the apprearance of the 28,500 band a diffuse low molecular band is seen in the gels (fig. 4.2 and 4.5). Degradation products of proteins other than rhodopsin may to some extent also contribute to the latter band. The 28,500 fragment still bears a carbohydrate group as intact rhodopsin does (Heller, 1968b). The 28,500 fragment obtained by pronase treatment also still contains the original chromophore binding site, as evident from the fluorescence found in this band after reductive fixation of the retinylidene-imine bond.

The 28,500 fragment is apparently consecutively degraded to a 21,500 fragment, when incubation is performed with pronase, subtilisin

or α-chymotrypsin (cf. fig. 4.2 and 4.5). Papain does not give rise to further digestion, which has also been reported by Trayhurn et al. (1974a). The 21,500 fragment also contains an oligosaccharide residue and for pronase treated preparations it is shown that the original chromophore binding site is still preserved. This indicates that the 21,500 fragment does indeed derive from further digestion of the 28,500 fragment and seems to exclude the possibility that these two major fragments are formed simultaneously. However, complete conversion of the 28,500 fragment into the 21,500 fragment has never been achieved, although α-chymotrypsin seems the most potent enzyme to form the latter product.

It is rather striking that a similar digestion pattern is found for a highly specific protease like α-chymotrypsin on the one hand and for subtilisin and pronase with their very broad specifity on the other hand. It should be born in mind, however, that there is no direct proof that the 28,500 fragments obtained by treatment with different enzymes are identical, while the same can be said for the 21,500 fragments. Moreover, the fact that all major fragments are glycoproteins may lead to incorrect molecular weight estimations (cf. Frank and Rodbard, 1975). Nevertheless, the equality of the apparent molecular weights of the fragments obtained with the different enzymes seems to justify the conclusion that rhodopsin is hydrolyzed by most proteases within a restricted region of the protein chain.

From the observations at this stage one might postulate a rather simple picture of sequential events during proteolysis. In the intact membrane rhodopsin is attacked by all proteases (except trypsin) and an 8000 MW fragment, which probably contains the phosphorylation site, is broken off. No property of rnodopsin is affected other than the protein chain length, and the phosphorylation site. The 8000 MW fragment is further degraded. Subsequently, a fragment with MW of about 7000 can be split off from the remaining part of tne rhodopsin molecule by pronase, subtilisin or α-chymotrypsin. This peptide may remain greatly intact since no increase in the amount of solubilized NH_2-compounds occurs during this step (second pronase addition of fig. 4.1).

Table 4.2

NUMBER OF PROTEIN BANDS OBSERVED IN ELECTROPHORETIC ANALYSIS OF PHOTORECEPTOR MEMBRANES TREATED WITH PROTEASES*

authors / protease	This thesis (bovine)	Saari, 1974 (bovine)	Trayhurn et al., 1974a,b,c (bovine)	Pober & Stryer, 1975 (bovine)	Klip et al., 1976 (bovine)	Aton & Barr, 1976 (frog)	Vandenberg et al., 1976 (frog)
pronase	3	n.d.	1	n.d.	4	n.d.	n.d.
papain	2	n.d.	2	2	n.d.	n.d.	n.d.
thermolysin	n.d.	1	n.d.	2	2	n.d.	n.d.
subtilisin	3	5	n.d.	2	n.d.	n.d.	3
α-chymotrypsin	3	4	0	2	2	n.d.	n.d.
trypsin	0	0	0	n.d.	n.d.	$\geq$ 2	0

*If its presence is reported, the protein band of small fragments in the tracking-dye region is included, the possibly residual opsin band is excluded.

n.d. = not determined.

Table 4.3

APPARENT MOLECULAR WEIGHT OF THE LARGEST PROTEOLYTIC FRAGMENT OF RHODOPSIN

authors / protease	This thesis (bovine)	Saari, 1974 (bovine)	Trayhurn et al., 1974a,b,c (bovine)	Pober & Stryer, 1975 (bovine)	Klip et al., 1976 (bovine)	Aton & Barr, 1976 (frog)	Vandenberg et al., 1976 (frog)
pronase	28,500 (+, +)	n.d.	24,800 (+, +)	n.d.	25,000 (+,n.d.)	n.d.	n.d.
papain	28,500 (+,n.d.)	n.d.	24,800 (+, +)	30,000 (+, -)	n.d.	n.d.	n.d.
thermolysin	n.d.	29,000 (+, +)	n.d.	30,000 (+, -)	27,250 (+,n.d.)	n.d.	n.d.
subtilisin	28,500 (+,n.d.)	29,000 (+,n.d.)	n.d.	30,000 (+, -)	n.d.	n.d.	25,000 (+, -)
α-chymotrypsin	28,500 (+,n.d.)	29,000 (+,n.d.)	resistant	30,000 (+, -)	24,360 (+,n.d.)	n.d.	n.d.
trypsin	resistant	resistant	resistant	n.d.	n.d.	30,000 (-, +)	resistant

n.d. = not determined. When no proteolysis is observed, this is indicated by "resistant".
In parentheses the presence (+) or absence (-) of sugar residue and chromophore binding site, respectively.

Other reports in the literature (Trayhurn et al., 1974a, b, c, 1975; Saari, 1974; Pober et al., 1976, Vandenberg et al., 1976; Klip et al., 1976) lead to a less simple picture of rhodopsin proteolysis. All authors agree with each other and us that rhodopsin completely retains its 500 nm absorbance during proteolytic treatment of photoreceptor membranes. However, the analysis of the proteolytic products has led to rather contradictory results. A rough comparison of the results reported by different authors is presented in tables 4.2 and 4.3. For none of the enzymes does complete agreement exist over the resulting number of rhodopsin fragments and their molecular weights. Only for the fragment with a molecular weight closest to that of opsin do the estimated molecular weights agree fairly well. All authors, except for Aton and Barr (1976) report that this fragment is a glycoprotein (see table 4.3). Not all authors find that this large fragment also bears the chromophore binding site.

Even larger discrepancies exist for the other fragments. Not only the total number and molecular weights of those fragments vary, but there is also disagreement about the presence of carbohydrates and the chromophore binding site. Some authors, in accordance with our results, claim consecutiveness of fragment formation (cf. Saari, 1974; Vandenberg et al., 1976; Klip et al., 1976), while other authors (Pober and Stryer, 1975; Pober et al., 1976) assume simultaneous production of all fragments, which would imply that they are complementary to each other.

The disagreement between the results of various authors may be attributed in part to differences in incubation conditions, to unadequate staining of gels for proteins or carbohydrates, to the electrophoresis system used (cf. Pober and Stryer, 1975), to inadequate inactivation of enzymes before addition of SDS, to impurity of the preparations and certainly to species differences. The exact characterization of the different proteolytic products of rhodopsin can only be achieved by resolving the primary structure of rhodopsin and its possible fragments. However, sequencing of membrane proteins is difficult, primarily because of their poor solubility in aqueous

media. The primary structure of only few intrinsic membrane proteins has been solved sofar (cf. Tomita and Marchesi, 1976; Green and Flanagan, 1976; Marchesi et al., 1976). Despite the relative ease of obtaining pure rhodopsin, its amino acid sequence is still unknown.

Amino acid analysis of more or less purified fragments of rhodopsin does not reveal significant alterations in the hydrophobicity index (cf. Trayhurn et al., 1974b; Saari, 1974; Pober and Stryer, 1975). It is, therefore, not even possible to conclude that proteolysis of rhodopsin in the intact membrane results in the removal of the hydrophilic, water exposed parts of the protein, as is generally accepted for other intrinsic membrane proteins.

4.4.2. Subunit structure?

Although we find that the major fragmentation products of rhodopsin (28,500 and 21,500) carry the chromophore binding site (see 4.3.1), this does not necessarily mean that the fragments also bear the complete chromophoric centre. In the membrane after protease treatment the entity responsible for the characteristic rhodopsin spectrum might consist of an artificial subunit structure· a large fragment (28,500 or 21,500) combined with one or more smaller peptides. At the SDS concentrations employed in electrophoretic analysis of the fragments, the rhodopsin spectrum is inevitably lost and if a subunit structure exists in the membrane it will not be revealed by SDS gel electrophoresis.

Treatment with papain results in one major products with MW 28,500 (cf. fig. 4.5). Trayhurn et al. (1974b) have reported the purification of this fragment without loss in rhodopsin absorbance, which would imply that this fragment contains the complete chromophoric centre. However, the same authors report that this fragment falls apart in smaller peptides, when SDS gel electrophoresis is performed in the presenc of β-mercaptoethanol (Trayhurn et al., 1974c). The 28,500 fragment would then consist of subunits linked together by S-S bridges. Since SDS gel electrophoresis in our study

has been performed under reductive conditions, our results do not confirm this finding.

When rhodopsin, after treatment of the intact membrane with thermolysin, is solubilized in detergent and bound to concanavalin A columns, illumination results in the release of a fragment which does not bear carbohydrates, but contains the chromophore binding site, while another fragment remains bound to the column. Similar results are obtained with α-chymotrypsin, papain and subtilisin (Pober and Stryer, 1975).

Electrophoresis of rhodopsin in the presence of the cationic detergents cetyl- of dodecyl-trimethylammonium bromide preserves its spectrum. Our preliminary results (not published) show that protease-treated rhodopsin moves in this system as a single band with an intact rhodopsin spectrum. Illumination then gives rise to further fragmentation and protein bands are found with much higher mobility (lower MW) than that of the non-illuminated sample.

These results and those of Pober and Stryer (1975) strongly suggest that proteolysis of rhodopsin has led to the formation of artificial subunits, which in detergent are held together by 11-cis retinaldehyde binding and not by S-S bridges, since the presence or absence of β-mercaptoethanol makes no difference. In the membrane the organization of this artificial subunit structure does not seem to depend on the presence of 11-cis retinaldehyde, since proteolyzed opsin or illuminated proteolyzed rhodopsin are both able to regenerate with 11-cis retinaldehyde.

These observations indicate that during proteolysis some fragments are formed simultaneously, while consecutive formation of proteolytic fragments has been concluded from the SDS gel electrophoretic analysis (see section 4.4.1). These contradictory results cannot yet be satisfactorily explained, but there seems to be a real possibility that none of the proteolytic products can independently provide the complete chromophoric centre.

4.4.3. Opsin proteolysis

SDS gel electrophoresis indicates that proteolytic treatment of illuminated rod outer segment membranes results in the same fragmentation pattern as found for non-illuminated membranes. This indicates that opsin is equally accessible to proteolytic attack as rhodopsin.

These results imply that photolysis of rhodopsin in the membrane is not accompanied by a major change in position of peptide bonds, which makes them susceptible to proteolytic action. This suggests that only relatively minor conformational changes take place upon photolysis in the intact membrane, a suggestion supported by sulfhydryl modification studies (De Grip et al., 1973a) and by measurements of circular dichroism (Shichi, 1971) and birefringence (Liebman et al., 1974) of rod outer segment membrane suspensions. Recent X-ray studies point in the same direction (Chabre, 1975).

4.4.4. Proteolytic effects on other membrane proteins

Small amounts of membrane proteins other than rhodopsin are detectable on SDS polyacrylamide gels. They invariably show higher molecular weights than opsin (see also Papermaster et al., 1976a and b). The gel pattern and quantitative data on the release of amino compounds indicate that those proteins are easily digested by proteolytic enzymes (cf. also Saari, 1974). The activity of the particulate retinoldehydrogenase, which is an entity distinct from rhodopsin (Etingof et al., 1972), is indeed extremely susceptible to proteolytic attack, even by trypsin, which leaves rhodopsin unchanged. Hence, these membrane proteins appear to have a rather accessible location in the membrane.

4.4.5. Proteolysis after membrane disruption

From the results of proteolysis of rod outer segment membranes it is clear that the susceptibility of rhodopsin to proteases is limited. Which factors determine this limited susceptibility? A more

extensive hydrolysis of peptide bonds and loss of spectral integrity is observed with all five proteolytic enzymes used in the present study for rhodopsin solubilized in four different detergents. Comparable results have previously been obtained by Radding and Wald (1958) for high concentrations of α-chymotrypsin in digitonin. The extensive hydrolysis of rhodopsin in detergent solution, shows that the limited extent of digestion in the membrane is not caused by mere inability of the enzymes to hydrolyse this protein. Since these detergents do not appreciably affect the activity of the enzymes (results unpublished), the effect of detergents on the proteolytic susceptibility of rhodopsin seems primarily the result of their making the pigment molecule more accessible to proteases. This accessibility increases in the order digitonin < Triton X-100 < Emulphogene BC-720 < CTAB. A similar sequence is found for the effects of these detergents on other properties of rhodopsin, e.g. the thermal stability (cf. fig 4.6.B) and the freedom of motion of rhodopsin parts as revealed by electron spin resonance studies (Pontus and Delmelle, 1975a). The regeneration capacity and the accessibility of sulfhydryl groups are also dependent on the detergents in this order (Daemen, 1974; De Grip, 1974), which parallels the solubilizing ability of these detergents towards the erythrocyte membrane (Kondo et al., 1972; cf. also Helenius and Simons, 1975; Tanford and Reynolds, 1976).

The detergent effects on rhodopsin can be interpreted in two ways: as resulting from disruption of lipid-lipid and lipid-protein interactions, leading to increased exposure of the rhodopsin molecule to the aqueous environment. Or else, the detergent could penetrate the rhodopsin molecule and partially unfold it, thus allowing a progressive proteolytic attack. In order to distinguish between these two possibilities, lipid- and detergent-free, pure rhodopsin has been exposed to pronase. Again extensive proteolysis occurs accompanied by loss in 500 nm absorbance (cf. fig. 4.8). This is not quite conclusive proof for the assumption that lipid removal renders rhodopsin susceptible to proteolytic attack, since detergent solubilization has been used in the purification of the rhodopsin

(see 3.3.7). This may conceivably have caused structural deformation of the rhodopsin, since there are also changes in photolytic behaviour (chapter 6), thermal stability and regeneration capacity (chapter 7).

Treatment of rod outer segment membranes by phospholipase C avoids the intermediate contact with detergents and still results in the effective removal of most of the phospholipids from the membrane (see chapter 8). In this case there is also increased susceptibility of rhodopsin to proteolytic attack. Although the enzymatic removal of phospholipids may lead to similar structural defects of rhodopsin as found for its lipid- and detergent-free state (chapter 6, 7 and 8), this experiment shows that the presence of a lipid bilayer is required for keeping rhodopsin only partially accessible to proteases, presumably because the lipids of the native membrane shield essential parts of rhodopsin. This indicates that this intrinsic membrane protein is embedded to a great extent within the hydrophobic core of the lipid bilayer of photoreceptor membranes. The next chapters will deal with the question to which extent the micro-environment is essential for the proper functioning of rhodopsin.

4.4.6. Conclusions

Proteolysis of rhodopsin in the intact membrane is greatly restricted and does not affect essential properties of the visual pigment. A consecutive partial degradation occurs, probably accompanied by simultaneous formation of a subunit structure. Presumably under these conditions a subunit arrangement rather than a single peptide constitutes the chromophoric centre of rhodopsin. The presence of an intact lipid bilayer is required for the maintenance of the limited proteolytic susceptibility of rhodopsin. This means that extensive and essential parts of rhodopsin are embedded within the hydrophobic core of the membrane. Illumination does not change the proteolytic susceptibility. Membrane proteins other than rhodopsin seem to be present in a rather accessible location in the membrane.

CHAPTER 5

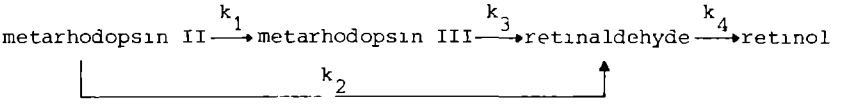
METARHODOPSIN II DECAY IN ROD OUTER SEGMENT MEMBRANES

5.1. Introduction

The sequence of spectral changes in rhodopsin following illumination is a very specific property of the visual pigment. The late and slower part of this photolytic sequence at temperatures between 4 and 37°C is accessible to spectrophotometry on a time scale of minutes. The metarhodopsin I to II transition is generally believed to involve significant protein conformational changes of the pigment (see section 1.4.3), whereas during the decay of metarhodopsin II retinaldehyde leaves its original binding site (Bonting et al., 1973; Rotmans et al., 1974). Both processes might very well be influenced by the micro-environment of the pigment molecule. Hence, a more detailed study of these aspects would seem to be useful.

The decay of vertebrate metarhodopsin II has primarily been studied in digitonin solution and in the intact retina. In 2% digitonin between pH 5.5 and 7.5 metarhodopsin II directly decays for about 30% to free retinaldehyde (λ_{max} = 380 nm), while the rest is converted to a product with λ_{max} = 465 nm, called pararhodopsin or metarhodopsin III (cf. Matthews et al., 1963, Ostroy et al., 1966). Protonated retinylidene-opsin (λ_{max} = 440 nm) may become involved at pH < 5.5, while its unprotonated equivalent is found at pH > 7.5 (Ostroy et al., 1966). The shift in λ_{max} from 380 to 465 nm during the conversion of metarhodopsin II to metarhodopsin III indicates protonation of the retinylidene-imine group during this step. Nevertheless, the transition is accompanied by the net loss of one proton per protein molecule (Ostroy, 1974).

Studies of isolated frog and human retinas, at high levels of illumination (Bauman, 1972, 1973), have yielded a quantitative description of the decay route of metarhodopsin II, according to the following scheme:



The reduction of retinaldehyde to retinol is only found when NADPH is available for the retinoldehydrogenase present in the photoreceptor membranes. The kinetics of the transitions imply that in frog and human retinas also about 30% of metarhodopsin II hydrolyses directly to retinaldehyde. This scheme has been confirmed by other authors (Gyllenberg et al., 1974; Paulsen et al., 1975). At more physiological levels of rhodopsin illumination (< 20% photolysis), the reaction rates of the transitions seem to be dependent on the Ca^{2+} concentration (Backstrom et al., 1974). The scheme is then probably no longer valid due to the appearance of a compound, designated as "bound retinaldehyde" (λ_{max} = 380 nm), at the expense of metarhodopsin III (Donner and Hemila, 1975).

Experimental modification of the micro-environment of rhodopsin is not readily performed in these systems. This is obvious for the intact retina where, in addition, the measurement of other rhodopsin parameters becomes complicated or impossible. In digitonin solution, rhodopsin is incorporated in detergent micelles: an artificial micro-environment by itself. Isolated rod outer segment membranes offer a system which allows experimental modification of the micro-environment, as well as the simultaneous determination of the various parameters. Since little information is available on the metarhodopsin II decay in bovine photoreceptor membrane suspensions, a more detailed characterization is presented in this chapter. In chapter 6 we shall use these characteristics to detect the effects of changing the micro-environment of rhodopsin.

We have used aqueous suspensions of washed bovine photoreceptor membranes to eliminate interference from enzymatic retinol produc-

tion, since endogenous NADPH is removed during isolation of the membranes. In order to avoid complications occurring upon variation of temperature, pH and degree of photolysis (see above), we have carried out all measurements at 25°C and pH 6.5, and rhodopsin photolysis of more than 65%.

5.2. Methods

5.2.1. Measurement of spectral transitions

Rod outer segment membranes are obtained as described in section 3.3.1 with omission of the enrichment step. The water washed membranes are resuspended in an appropriate volume of the desired buffer, usually 0.1 M phosphate buffer (pH 6.5), at a concentration of 10 to 30 μM rhodopsin.

Spectral measurements of these rhodopsin preparations are performed at 25°C in a Rapid T3 spectrophotometer (see section 3.3.2). The spectra are recorded from long to short wavelengths at a scanning-speed of 4 or 6 nm/sec. Illumination is performed with a Rollei Strobofix flasher equipped with a 3 mm thick OG_{530} filter (Schott-Jena, Mainz, W. Germany) or for 20 or 30 seconds with a 300 W tungsten source at a distance of 25 cm behind a 3mm OG_{530} filter. Spectra are recorded before illumination and at various time intervals thereafter. The actual time interval after illumination at each wavelength is calculated from the scanning-speed, knowing the wavelength at which recording has been started, and the time elapsing between illumination and beginning of recording.

After recording of the spectral transitions hydroxylamine (final concentration 50 mM) is added to the suspension, which completely converts all photoproducts to retinylidene-oxime in 10 min. Another spectral recording is then made, which gives the residual rhodopsin (spectrum 15 in fig. 5.3). A subsequent exposure of the suspension to filtered light (OG_{530} filter) for 5 minutes completely photolyzes the residual rhodopsin and again a spectrum is recorded

(spectrum 16 in fig. 5.3). The fraction (A) of rhodopsin which has not been illuminated during the first light exposure is calculated from these two spectra and the spectrum of the suspension before illumination (spectrum 1 in fig. 5.3).

5.2.2. Determination of retinaldehyde and its Schiff bases

The relative concentrations of free retinaldehyde and its Schiff bases are determined by means of a spectrophotometric method described by De Pont et al. (1970b). The A_{440}/A_{380} ratio is measured after adding first 100 µl 2 M HCl to 1 ml of the (illuminated) rhodopsin preparation and then 100 µl Triton X-100 (10%, w/v) to obtain a clear solution.

Under these conditions the absorbance maximum of free retinaldehyde remains at 380 nm, while all Schiff bases of retinaldehyde are converted to their stable protonated form (cf. De Pont et al., 1970b). The absorbance maxima of all protonated retinylidene-imines, derived from aliphatic amines, are identical at 440 nm. The molar absorbances of these imines are also identical. Acidification of (residual) rhodopsin leads to its denaturation and the protonated imine of 11-cis retinaldehyde is formed with λ_{max} = 440 nm, but with a lower molar absorbance than that of its all-trans isomer. The 11-cis isomer is apparently rather labile and is gradually converted to the all-trans form. Errors due to the presence of varying and unknown fractions of 11-cis retinaldehyde derivatives can be eliminated by illumination of the acidified, Triton X-100 solubilized preparation before determination of the A_{440}/A_{380} ratio. After exposure of the preparation to white light for 5 to 10 minutes (300 W tungsten source at a distance of 40 cm) the molar absorbance of all the retinylidene-imines appears to be equal.

A simple nomogram can now be used (cf. De Pont et al., 1970b). In fig. 5.1 the percent of (protonated) Schiff base of retinaldehyde is plotted against the A_{440}/A_{380} ratio of mixtures of retinaldehyde and retinylidene-dodecylamine in acidified Triton X-100 solution. Exactly the same nomogram is obtained with retinaldehyde and retinyl-

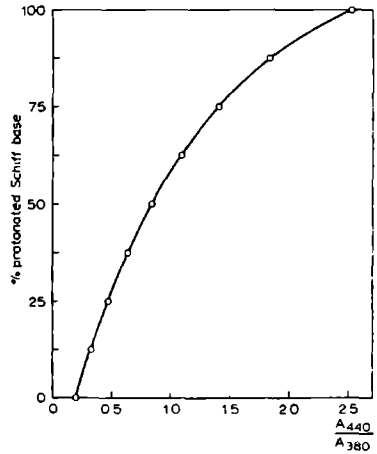


Fig. 5.1. Relationship between the A_{440}/A_{380} ratio and the percentage of protonated retinylidene-dodecylimine in mixtures of this compound and retinaldehyde in acidified Triton X-100 solution.

idene-imines of octylamine and phosphatidylethanolamine, prepared as described in section 5.2.4. Illumination of the mixtures does not have any effect on the nomogram. Thus, for any A_{440}/A_{380} value measured in acidified, Triton X-100 solubilized and illuminated preparations, the percent Schiff base or free retinaldehyde is easily found by this nomogram. Since we are interested in the concentrations of free and bound retinaldehyde relative to the total concentration of photoproducts, a correction must be made for the contribution of retinylidene-imines derived from residual rhodopsin. If A is the fraction of residual non-illuminated rhodopsin (see section 5.2.1), the corrected percent free retinaldehyde is $\frac{1}{1-A}$ times the free retinaldehyde obtained from the nomogram.

5.2.3. Discrimination between lipid and protein bound retinylidene-imines

In the previous section a procedure is described to determine the fraction of retinylidene-imines in the photoproducts of rhodopsin.

This procedure does not discriminate between retinylidene-imines of proteins or lipids. Such a discrimination can be achieved by a lipid extraction procedure (see fig. 5.2).

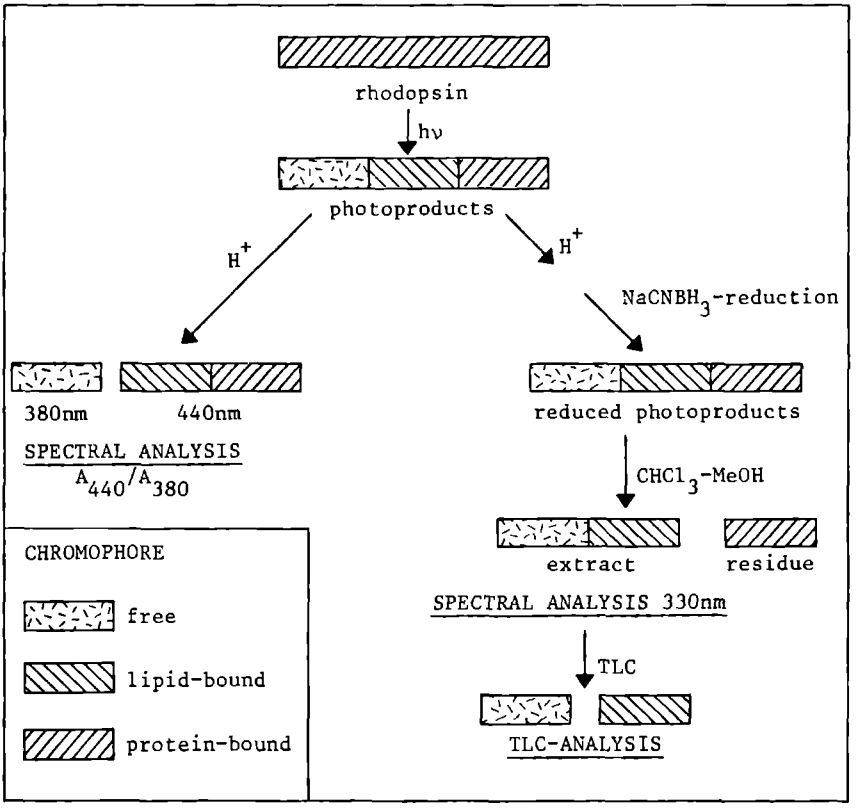


Fig. 5.2. Schematic presentation of photoproduct analysis. The composition of photoproducts is analyzed either by two-wavelength spectrophotometry after acidification (free chromophore vs. protonated aldimine, section 5.2.2), or by reduction and subsequent spectral analysis at 330 nm of the $CHCl_3$-MeOH-extractable chromophore (free + lipid-bound, section 5.2.3), incidently followed by chromatographic analysis (free vs. lipid-bound, section 5.2.5). The figure is not intended to suggest a realistic quantitative analysis.

Extraction of protonated retinylidene-imines by chloroform-methanol mixtures gives poorly reproducible results, due to Schiff base deprotonation in the organic phase. An irreversible fixation

of the Schiff bases can be achieved by reduction with $NaCNBH_3$, which converts the imines to secondary amines (see fig. 1.7). Sodium cyanoborohydride is used, since it operates at low pH values (Borch et al., 1971), in contrast to sodium borohydride.

To 4 ml of the (illuminated) rhodopsin preparation 0.4 ml 2 M HCl is added under vigourous agitation, and 4 ml of the suspension is centrifuged for 30 minutes at 4°C (5,000 rpm, Sorvall RC2B, rotor SS34). After removal of 3.0 ml of the clear supernatant, 3.8 ml of chloroform-methanol (1:2, by volume), containing 10 mM BHT (butylated hydroxytoluene), is added to the residue. All material, including the protein, dissolves in the homogeneous solution (4.7 ml). Sodium cyanoborohydride (5-10 mg) is added to this clear solution, and instantaneous reduction of all free retinaldehyde to retinal and protonated Schiff bases to retinyl-amines is observed. From the absorption of the clear solution (A_{cs}) of reduced compounds at 330 nm the total chromophore concentration is calculated. Then 1.2 ml $CHCl_3$ and 1.2 ml 0.1 M KCl are added to the solution to obtain separation between an organic (lower) and an aqueous (upper) phase. The mixture is shaken 10 minutes in sealed tubes under a nitrogen atmosphere. It is then centrifuged for 10 minutes at room temperature (5,000 rpm, Sorvall RC2B, rotor SS34) to accelerate separation of the phases. About 1.5 ml of the organic lower phase (2.35 ml total) is removed to measure the 330 nm absorbance (A_{op}). The proteins are assembled in a thin layer at the boundary of the two phases. The upper phase is invariably free of compounds absorbing between 300 and 600 nm. All manipulations are carried out in darkness. BHT, an antioxidant, is added to prevent absorbance loss by oxidation of retinol or retinyl-compounds.

The fraction of the chromophore which can be extracted by this procedure can be calculated from the molar absorbance of the compounds, the absorbance of the clear solution (A_{cs}) and that of the lower phase (A_{op}) and the volume ratio between both solutions. The molar absorbance and the absorbance maximum of all-trans retinol are identical to those of all-trans retinylamines (ε = 51,000), but in the clear solution also 11-cis retinyl-compounds derived from

residual rhodopsin contribute and their molar absorbance is 0.62 times that of the all-trans form. Correction for this can be made from the known fraction of residual rhodopsin (A) after illumination of the suspension (see section 5.2.1).

To obtain the percent of extractable compounds relative to the sum of photoproducts, the contribution of the retinylamines derived from residual rhodopsin must be eliminated, which can be achieved by a calculation analogous to the one described at the end of the previous section.

Several control experiments, using thin layer chromatography for product detection (see section 5.2.4), show that in the procedure described here no additional Schiff bases are formed after acidification, and that no transiminization occurs. The reduction by $NaCNBH_3$ is fast and is not accompanied by a troublesome increase in pH, since the pH of the aqueous upper phase never exceeds 4. Other control experiments with retinaldehyde and (protonated) retinylidene-imines of phosphatidylethanolamine and dodecylamine show a quantitative recovery of the reduced compounds. In every experiment a control, consisting of the acidification, reduction and extraction of non-illuminated rhodopsin preparations is carried out to see that all retinaldehyde has remained bound to the protein. This is indeed the case, since never material absorbing beyond 300 nm is extracted.

The procedure of section 5.2.2 yields the fraction of free retinaldehyde and the present procedure the total extractable fraction. This gives by substraction the fraction of lipid-bound retinylidene-imine (cf. fig. 5.2).

5.2.4. Preparation and identification of retinylidene-imines and retinylamines

The protonated retinylidene-imines, which are used to obtain the nomogram (see section 5.2.2 and fig. 5.1), are prepared by combining a three-fold molar excess of the amine (dodecyl-, octyl- or phosphatidylethanol-amine) with retinaldehyde in absolute methanol.

Pyridine is added to a final concentration of about 5% (v/v). After evaporation of the methanol, 0.1 M HCl in 1% (w/v) Triton X-100 is added. In this way all retinaldehyde is converted into its stable protonated retinylidene-imine. The excess of the amines does not cause spectral interference.

In order to check whether transiminization or additional Schiff base formation occurs after the acidification, reduction and extraction described in section 5.2.3, the retinylidene-imines have also been prepared with an excess of retinaldehyde. About 300 nmol dodecylamine, phosphatidylethanolamine or phosphatidylserine are solubilized in methanol containing 1 μmol retinaldehyde. The solution is evaporated to dryness and 100 μl absolute methanol is added, followed by 500 μl 0.1 M KCl and 15 μl of 2 N NaOH, and finally after 10 minutes 100 μl of 2 N HCl. The mixture is then dissolved in 1.9 ml of chloroform-methanol (1:2 by vol.). After addition of 0.6 ml of 0.1 M KCl and 0.6 ml $CHCl_3$, the resulting organic phase contains most of the unreacted amines as well as all the retinaldehyde and protonated imines. If NaCNBH has been added to the chloroform-methanol solution, the organic phase contains the reduced forms of the reaction products (see section 5.2.3). The protonated imine of retinaldehyde and phosphatidylserine is not easily formed, but its yield is increased by the presence of 50 mM $CaCl_2$ in the 0.1 M KCl solution. In addition, it appears that in the organic underphase this imine decomposes within 1 hr.

Extractable protonated retinylidene-imines can be identified by thin layer chromatography on silicagel G plates (Merck; 0.25 mm), which have previously been immersed in a 1% solution of oxalic acid and dryed and reactivated at 100°C for 30 minutes. Oxalic acid prevents transiminization during application of the samples on the silicagel plates. The plates are developed with chloroform-metnanol--12 N HCl (120:9:1, by volume). The Rf values of the protonated retinylidene-imines of dodecylamine, phosphatidylethanolamine and phosphatidylserine are 0.43, 0.13 and 0.08 respectively, while retinaldehyde migrates near the front (Rf = 0.96). The protonated retinylidene-imines are easily detected by their characteristic

orange colour. Retinaldehyde is yellow and the amines (Rf < 0.05) are detected by ninhydrin.

Chromatography shows that no transiminization of formation of retinylidene-imines occurs after acidification, since addition of retinaldehyde and/or amines to acidified retinylidene-imines does not result in new retinylidene-imines. In a similar way the cyanoborohydride reduction procedure could be shown to be correct (compare Daemen et al., 1971).

5.2.5. Chromatographic estimation of retinylamines

The identity of the retinylamines obtained by reduction with $NaCNBH_3$, as described in section 5.2.3, can be estimated by thin layer chromatography. The only extractable retinylamines which have been formed, are those of phosphatidylethanolamine or phosphatidylserine, the only two phospholipids with free amino groups. Chromatography is performed on glass plates (20 x 20 cm), coated with a 0.3 mm thick layer derived from a mixture of purified silicagel H (Merck) and 4% alkaline magnesium silicate (cf. Broekhuyse, 1968). A known volume of the organic phase, containing the extractable retinylamines and retinol (see section 5.2.3) is quantitatively applied to the thin layer plate. Chromatography is performed in chloroform-methanol-acetic acid (90:15:6, by volume). Retinylamines and retinol can be detected by their fluorescence, when excited by ultraviolet light. All compounds can be detected by iodine vapor. Retinol migrates near the front, while retinylphosphatidylethanolamine (Rf = 0.75) is completely separated from phospholipids (Rf < 0.45), but not retinylphosphatidylserine (Rf = 0.5). Since the latter compound appears to be hardly present in out preparations, no attempt has been made to improve the separation.

The retinylphosphatidylethanolamine can be quantitatively estimated by phosphate determination. About 60 nmol of the substance is needed for this purpose. Since only about 20% of the photolytic products of rhodopsin in rod outer segment membranes appear to yield this compound, about 300 nmol of illuminated rhodopsin has to be acidified, reduced and extracted. The extract then contains about

20 µmol of phospholipids and this 300-fold excess of phospholipids over retinylphosphatidylethanolamine requires distribution of the extract over about 15 spots to prevent overloading of the thin layer plate. BHT is omitted during the present procedure, since it interferes the chromatographic separation of retinol and retinylphosphatidylethanolamine. Therefore, the entire procedure is performed in darkness under a nitrogen atmosphere. Reference compounds are prepared as described in section 5.2.4. The retinylphosphatidylethanolamine spots and some blank areas are scraped from the thin layer plate for determination of the phosphate content after acid destruction (see section 3.3.6). Calculation of the retinylphospholipid content in the photolytic products of rhodopsin is based on this phosphate determination after chromatography and on the total chromophore content before extraction (A_{cs} in section 5.2 3), applying the corrections for interference of residual rhodopsin as described in section 5.2.3.

5.3. Results

5.3.1. Spectral characterization of the metarhodopsin II decay

A typical example of the spectral transitions observed in illuminated suspensions of rod outer segment membranes at 25°C (pH 6.5) is shown in fig. 5.3. Within the time-scale used, rhodopsin (fig. 5.3 spectrum 1) is upon illumination immediately converted to a component with maximal absorbance at about 380 nm (fig. 5.3 spectrum 2, fig. 5.4.A), which is called metarhodopsin II (cf. Matthews et al., 1963). Subsequently there is a gradual decrease of the 380 nm absorbance concomitantly with the appearance of a component (metarhodopsin III) absorbing maximally at about 455 nm (fig. 5.3, spectra 2 to 14, fig. 5.4.B). After 40 minutes the spectrum remains fairly stable for at least 1 hour, indicating that no further spectral transitions occur. The isosbestic point lies at 417 nm.

Since we know the time at which the spectra are recorded, the absorbance increase or decrease ($A_t - A'_o$) can be plotted against

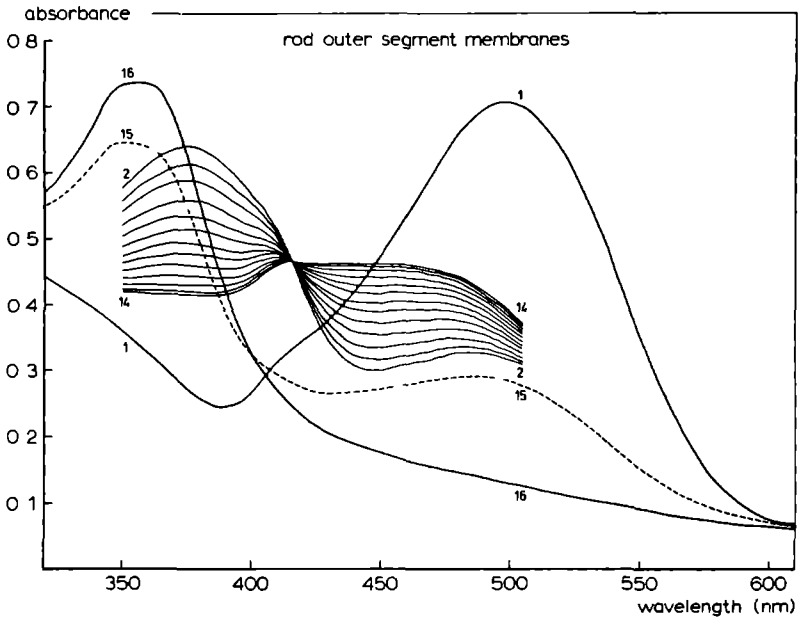


Fig. 5.3. Decay of metarhodopsin II after flash-photolysis of a rod outer segment membrane suspension in 0.1 M phosphate (pH 6.5) at 25°C. Every spectrum is recorded with a scanning speed of 4 nm/sec, starting at the long wavelength. The stable rhodopsin spectrum (1) is obtained before illumination. At time zero the suspension is illuminated for 2 msec with a flash-gun, equipped with an OG_{530} filter. The recordings of spectra 2 to 14 are started from 505 nm at the following times (in minutes after the flash)· 0.48; 1.64; 2.81; 4.06; 5.39; 6.73; 8.23; 9.81; 11.56; 13.81; 16.64, 19.52; 21.06. Subsequently, hydroxylamine is added to a final concentration of 50 mM, and 10 min later spectrum 15 is recorded, yielding a spectrum of residual rhodopsin and retinylidene-oxime (λ_{max} = 365 nm). A second light exposure for 5 minutes abolishes all rhodopsin, yielding spectrum 16. From the absorbance at 500 nm of spectra 1, 15 and 16, the residual rhodopsin can be calculated, which in this case is 29.1%. For further details see section 5.2.1.

time, taking the start of illumination as zero-time. This is done in fig. 5.5.A for the data in fig. 5.3 at 380 and 440 nm, taking spectrum 2 as the arbitrary zero-level (A'_o). The absorbance difference at infinite time ($A_\infty - A'_o$) can be approximated by extrapolation of the curves of fig. 5.5.A. However, a more accurate value is found

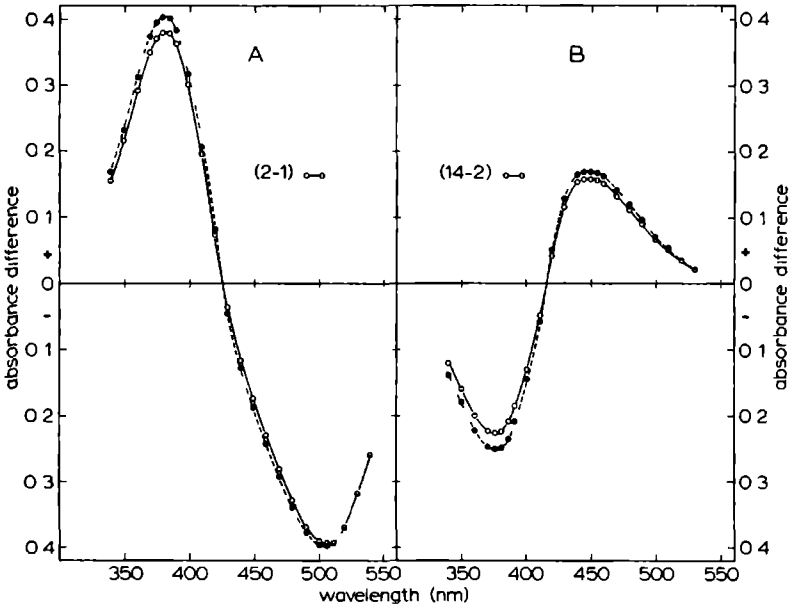


Fig. 5.4. Difference spectra obtained from the data of fig. 5.3. The solid curves in A and B represent the differences in absorbance between spectra 2 and 1 and between spectra 14 and 2 respectively. In order to correct for the influence of the time delay in the recording of spectrum 2, the spectrum at zero-time of the transition has been obtained by calculated extrapolation, using the first order kinetic description of the spectral transition (see text and fig. 5.5). Taking spectrum 1 and 14 as time-invariant, the absorbance differences between each of these two spectra and the calculated spectrum at zero-time of the transition yield the dashed curves in A and B respectively.

by employing a least square method, assuming first order kinetics: $\ln\left[(A_\infty - A_t)/(A_\infty - A'_o)\right] = -k.(t - t'_o)$. Various values of A_∞ are tested to find the value giving the smallest sum of least squares, when the logarithm of $(A_\infty - A_t)/(A_\infty - A'_o)$ is plotted against time, as is done in fig. 5.5.B for the data of fig. 5.3. Commonly, for every kinetic analysis the absorbance differences $(A_t - A'_o)$, belonging to 6 or more time intervals after the onset of illumination, are used, taking care that they comprise more than 80% completion of the

transition. In all cases tested (n = 29) the correlation coefficient obtained by the least square method, invariably lies above 0.9999, indicating that the transition obeys first order kinetics almost ideally.

Fig. 5.5 shows that the arbitrary zero point may deviate some-

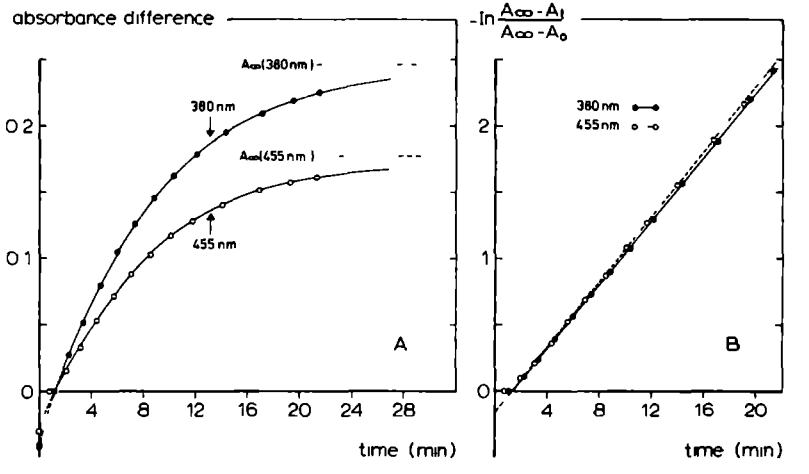


Fig. 5.5. Analysis of the absorbance changes at 380 and 455 nm during metarhodopsin II decay (data from fig. 5.3). A: absorbance change ($|A_t - A'_0|$) at 380 nm (decrease) and 455 nm (increase) plotted against time, taking the absorbance of spectrum 2 at these wavelengths as arbitrary zero level (A'_0). B: First order kinetic description of the absorbance changes at 380 nm (solid line) and 455 nm (dashed line): $\ln\left[(A_\infty - A_t)/(A_\infty - A'_0)\right] = -k.(t - t'_0)$. A_∞ is found by a least square method (see text) and the levels are indicated in part A. The actual absorbance level at zero-time (t_0) is found by (calculated) extrapolation of the straight lines (see B) and the result is indicated by the dashed parts of the curves in A. In this case the values of k (min^{-1}) are 0.120 and 0.121 for 380 and 455 nm respectively.

what from the ideal curve. This is occasionally found when the sample is flash-illuminated in the cuvette. It probably derives from incompleteness of the transition of metarhodopsin I (λ_{max} = 478 nm) to metarhodopsin II. If such effects are found, the coordinates of this point are not used in the least square analysis.

The kinetic constants of the rate of the absorbance decrease at 380 nm and increase at 455 nm are listed in table 5.1. They are

Table 5.1

KINETIC AND SPECTRAL CONSTANTS OF METARHODOPSIN II DECAY

absorbance change at:	k in min^{-1} (± SE)	$t_{\frac{1}{2}}$ in min (± SE)	number of determinations
380 nm	0.129 (± 0.003)	5.37 (± 0.12)	29
455 nm	0.131 (± 0.005)	5.29 (± 0.20)	29
$\frac{(A_\infty - A_o)_{380}}{(A_\infty - A_o)_{455}}$ = 1.49 (SE = 0.03)			29
metarhodopsin II : λ_{max} = 380 nm			
metarhodopsin III : λ_{max} = 455 nm			isosbestic point: 417 nm
free retinaldehyde: λ_{max} = 380 nm			

identical within the limits of detection, as is also the case at every other wavelength within the spectral range. This is not unexpected in view of the observed isosbestic point, which indicates that probably only two spectrally distinct components are involved in the transition.

Spectrum 2 (fig. 5.3) is arbitrarily chosen as zero-level for the kinetic analysis, but the spectrum at the real zero time of the transition (t_o) can be calculated using the kinetic constant k (see extrapolation in fig. 5.5). Since illumination itself takes time, the zero-time of the transition is best approximated by the midpoint of the time of illumination (Donner and Hemila, 1975). The calculated extrapolation to this zero-time gives, at any wavelength, the total difference in absorbance at time zero and infinite time ($A_\infty - A_o$). In this way the ratio between the total absorbance difference at 380 nm and at 455 nm is expressed by $(A_\infty - A_o)_{380}/(A_\infty - A_o)_{440}$ and is calculated to be about 1.5 (see table 5.1).

Difference spectra can be corrected by similar calculation.

Since the scanning of a spectrum takes about 1 minute, the difference spectra obtained from actual recordings are deformed by the time delay between the measurement at different wavelengths. This effect is eliminated if the kinetic constant is used to recalculate the recorded spectra to spectra that would have been found if the scanning time was reduced to zero. Difference spectra corrected in this way are shown in fig. 5.4 (dashed curves), taking spectra 1 and 14 (fig. 5.3) as time-invariant. The effects on the absorbance difference are obvious, but apparently the effect of time delay in the recording of the spectra hardly influences the position of the absorbance maxima. Therefore, it can be concluded that metarhodopsin II and metarhodopsin III indeed exhibit their maximal absorbance at 380 nm and 455 nm respectively.

The metarhodopsin II to metarhodopsin III transition in rod outer segment membrane suspensions is not influenced by glycerol (up to 30%, v/v), the ionic strength of the phosphate buffer (0.01 M to 0.2 M) or the additional presence of dithioerythritol (10 mM) or 0.1 M KCl, provided that it is measured at 25^{o}C and pH 6.5. The transition is not affected either by replacing the phosphate buffer by 0.1 M acetate buffer (pH 6.5) and by the presence of up to 20 mM Ca^{2+} or 10 mM EDTA in this buffer. The time of illumination also plays a small role, since illumination periods of up to 10 minutes do not exhibit any influence on the kinetics of the transition.

In these experiments always more than 65% of rhodopsin has been illuminated. It may be clear from fig. 5.3, that not all of the absorbance gain at 380 nm, immediately after illumination, is lost in the transition to metarhodopsin III. It can be calculated that approximately 30% of the total initial absorbance gain at 380 nm (at zero-time of illumination) persists after completion of the transition (40 minutes after illumination). This is an indication that not all metarhodopsin II is exclusively converted into metarhodopsin III.

5.3.2. Chemical characterization of the decay products of metarhodopsin II

The relative concentration of free retinaldehyde, lipid bound retinylidene-imines and protein-bound retinylidene-imines, at any time after the initial illumination, have been determined by means of the procedures described in sections 5.2.2 and 5.2.3. The formation of these compounds exhibits similar time-courses as found for the spectral transition, and all processes obey first order kinetics. In metarhodopsin II all retinaldehyde is protein-bound, since no photoproducts can be extracted if illumination is immediately followed by acidification and reduction. Products analysis after completion of the metarhodopsin II decay (40 minutes after illumination) shows that free retinaldehyde comprises about 34% of all photoproducts, while the lipid-bound and protein-bound retinylidene--imines account for 15% and 51% respectively (see table 5.2). These

Table 5.2

COMPOSITION OF PHOTOPRODUCTS AFTER METARHODOPSIN II DECAY

compound	percent of total photoproducts (± SE)	number of determinations
free retinaldehyde[1)]	33.8 (+ 1.3)	9
lipid bound retinylidene-imine[2)]	15.2 (± 0.6)	8
protein bound retinylidene-imine[2)]	51.0 (± 1.8)	8
retinylidene-phosphatidylethanolimine[3)]	18 (± 3)	2

1): determined according to section 5.2.2; 2): according to 5.2.3;
3): according to 5.2.5. Compare fig. 5.2.

values remain quite similar for 3 hours, indicating that no further transitions occur (except for possible equilibria between the compounds).

If acidification, reduction and extraction at 40 minutes after

illumination is followed by thin layer chromatography (see 5.2.5), phospholipids, retinol and retinyl-phosphatidylethanolamine can be identified as the major extractable compounds. Only a trace of retinyl-phosphatidylserine may be present, as detected by its fluorescence. This again shows that retinaldehyde is a substantial product of the metarhodopsin II decay. It also shows that lipid-bound retinaldehyde is almost exclusively retinyl-phosphatidylethanolamine. Its quantitative determination further substantiates that the lipid-bound fraction of photoproducts exists of retinylidene-phosphatidyl-ethanolimine (see table 5.2).

5.4. Discussion

As illustrated in fig. 5.3, a very characteristic and reproducible spectral transition is observed after illumination of a bovine rod outer segment membrane suspension (25^{o}C, pH 6.5). The transition exhibits first order kinetics ($t_{\frac{1}{2}}$ = 5.3 min) and is completed after about 40 min. At that time no metarnodopsin II remains. This conclusion derives from the observation (Rotmans et al., 1974) that rhodopsin regenerates nearly completely after reduction of all retinylidene-imines present after completion of the metarhodopsin II to III transition, when taking into account that in metarhodopsin II retinaldehyde is still bound to the original binding site. In addition, the present spectral observations also suggest that no metarhodopsin II has remained at 40 min. after illumination, since at that time the absorbance at 380 nm still accounts for about 30% of the initial absorbance gain at that wavelength. The concentration of free retinaldehyde after completion of the transition is about 30% of the initial metarhodopsin II concentration (see table 5.2). Thus, not metarhodopsin II, but free retinaldehyde likely accounts for the residual absorbance at 380 nm.

Our results show that metarhodopsin III exhibits maximal absorbance at about 455 nm and that the spectral transition has an isosbestic point at 417 nm. In frog retinas the isosbestic point

lies at 426 nm (Hanawa and Matsuura, 1975), but in this species metarhodopsin III has a higher λ_{max} (465-475 nm, cf. Gyllenberg et al., 1974). However, bovine metarhodopsin III in digitonin also has an absorbance maximum at about 465 nm, while the isosbestic point of the transition is found at 412.5 nm (cf. Matthews et al., 1963). This discrepancy with our results must be attributed to detergent effects, digitonin causing a bathochromic shift of λ_{max} of metarhodopsin III and a hypsochromic shift of the isosbestic point either through broadening of the metarhodopsin III peak or a sharpening of the metarhodopsin II peak. Digitonin also appears to destabilize metarhodopsin III, since in its presence this intermediate decays with a half-life time of about 20 min (cf. Baker and Williams, 1968), whereas in the aqueous membrane suspension used in our study it is virtually stable for 3 hr after illumination.

Although equilibria between the decay products of metarhodopsin II cannot be excluded, the observed spectral transitions in combination with the measurements of the product concentrations at the end of the transition, are most simply explained by assuming that metarhodopsin II has three simultaneous decay routes to fairly stable products. One route is the direct hydrolysis to retinaldehyde (34%), while the other two routes exist of a transiminization of retinaldehyde to either an aminogroup of the protein (51%) or of phosphatidylethanolamine (15%). Our quantitative estimation of free retinaldehyde confirms the conclusion of Baumann (1972, 1973) and Ostroy et al. (1966), based on spectral transition measurements only, that about 30% of metarhodopsin II directly hydrolyses to free retinaldehyde. However, the involvement of retinylidene-phosphatidylethanolimine has so far not been taken into consideration, although the possible occurrence of such a compound has been mentioned briefly by Abrahamson et al. (1974).

The scheme generally used to explain the spectral transitions accompanying the metarhodopsin II decay (see section 5.1), is based on computer-simulation of spectral changes at specific wavelengths (cf. Baumann, 1972). However, under our conditions the formation of free retinaldehyde from metarhodopsin III, included in this scheme,

is either a very slow process or does not occur at all. Furthermore, retinylidene-phosphatidylethanolimine must be incorporated into this scheme. Apparently it is indistinguishable from either metarhodopsin III or free retinaldehyde, because only one isosbestic point is observed, indicating the involvement of only two spectrally distinct products of metarhodopsin II. Our product analysis proves the existence of retinylidene-phosphatidylethanolimine, but does not discriminate between the protonated or unprotonated form, because acidification is used to stabilize the retinylidene-imines (compare fig. 5.2). Therefore, determining whether retinylidene-phosphatidylethanolimine contributes to the absorbance of free retinaldehyde or of metarhodopsin III must be done through spectral measurements. We find that during metarhodopsin II decay the total decrease in absorbance at 380 nm is about 1.5 times the gain at 455 nm. Based on measurements in digitonin solution (Matthews et al., 1963) it is generally accepted that rhodopsin, metarhodopsin II and III and retinaldehyde have equal molar absorbances at the absorption peak. If this also applies to our measurements in aqueous suspension and if we assume that the residual absorbance at 380 nm after completion of metarhodopsin II decay can be completely attributed to free retinaldehyde absorbance, a simple calculation learns that about 20% of the initial metarhodopsin II absorbance is not found in the spectral region between 300 and 600 nm. This would mean that retinylidene-phosphatidylethanolimine would not absorb at all. The situation becomes even more complicated, when one takes into account that metarhodopsin III certainly contributes to the residual absorbance at 380 nm. This would imply that we are even short in accounting for the absorbance of free retinaldehyde. Assuming a molar absorbance of metarhodopsin III smaller than that of metarhodopsin II would solve a large part of the problem. In any case, the present spectral measurements do not tell us whether retinylidene-phosphatidylethanolimine has its maximal absorbance at 380 or at 455 nm. This problem will be further discussed in chapter 6, where the formation of retinylidene-phosphatidylethanolimine is prevented by eliminating phosphatidylethanolamine from the membrane (see section 6.4.2).

Whatever the spectral contribution of the lipid-bound retinaldehyde may be, it is clear from spectral measurements and product analysis, that metarhodopsin III consists mainly of a protein-bound retinylidene-imine. Since metarhodopsin III can be converted to metarhodopsin II by flash-illumination, it has been suggested that metarhodopsin III derives from a Schiff base of 13-cis retinaldehyde (Matthews et al., 1963). However, our efforts to isolate and identify this isomer in metarhodopsin III did not result in any indication of its presence. The results of recent flash-photolysis experiments indicate that it is not necessary to postulate a 13-cis configuration in metarhodopsin III to explain its photoconversion to metarhodopsin II (Reuter, 1976). Further aspects of the nature of metarhodopsin III will be discussed in the next chapter.

We have shown that the metarhodopsin II decay in isolated rod outer segment membranes can be satisfactorily measured, both with regard to the spectral aspects and to the photoproduct analysis. Hence, these two characteristics can be used as parameters for the functional and structural integrity of rhodopsin. In the next chapter these parameters will be used to study the behaviour of rhodopsin in an experimentally changed micro-environment.

CHAPTER 6

PHOTOLYSIS OF RHODOPSIN IN A MODIFIED MICRO-ENVIRONMENT

6.1. Introduction

Modification of the micro-environment of rhodopsin is a useful approach to investigate the extent to which the structure and function of the protein depends on presence and composition of the lipid bilayer. The modification can be achieved in various ways and to different degrees. Conversion of phosphatidylethanolamine and phosphatidylserine to their acetylated analogues will change the polar head group composition of the phospholipids of the photoreceptor membrane. Phospholipase C treatment of rod outer segment membranes results in a removal of polar head groups of phospholipids and thus in a reduction of the amount of amphipathic lipids. Complete lipid removal by means of affinity chromatography and subsequent detergent dialysis results in aqueous suspensions of lipid- and detergent-free rhodopsin.

Replacement of the original lipids by various other lipids provides yet another way in which the micro-environment of rhodopsin can be altered. This is achieved by complete removal of lipids from rhodopsin and subsequent reassembly with selected lipids with or without the intermediate use of detergent. The detergent dialysis procedure results in the formation of artificial rhodopsin-lipid recombinant membranes, when amphipathic lipids are used. In this way rhodopsin can indeed be reconstituted into lipid bilayers (cf. Chen and Hubbell, 1973).

In the previous chapter we have characterized the later stage of the photolytic sequence of rhodopsin in its native membrane

environment. In the present chapter we will use these characteristicts to establish the effects of alterations in the micro-environment of rhodopsin. Especially the effects on metarhodopsin II decay and the consequences for the nature of metarhodopsin III will be discussed.

6.2. Methods

6.2.1. Preparations and spectral measurements

Rod outer segment membranes are isolated as described in section 3.3.1. Phospholipase C treatment* is performed according to section 3.3.10. Lipid-free rhodopsin* in detergent solution is prepared by affinity chromatography (see section 3.3.7), while lipid- and detergent-free rhodopsin* is obtained by detergent dialysis as described in section 3.3.8. In the latter section the procedure for the reassembly of rhodopsin and lipids by the detergent dialysis procedure is also presented. Mere addition of lipids to rhodopsin preparations, without the intermediate use of detergent, is performed according to section 3.3.9.

Section 5.2 includes the procedures for spectral measurements, which are performed at 25°C in 0.1 M phosphate buffer pH 6.5, and analysis of the photoproducts. In some preparations glycerol (up to 30% v/v) is added to improve the stability of the suspensions; its presence does not influence the results.

*Terminology:
1. Photoreceptor (or rod outer segment) membranes exhaustively treated with phospholipase C the membrane preparation in which about 90% of the phospholipids is hydrolyzed by this enzyme.
2. Lipid- and detergent-free rhodopsin: rhodopsin suspension obtained after lipid removal by affinity chromatography in DTAB solution and subsequent removal of the detergent by dialysis.
3. Delipidated rhodopsin preparations: collective noun for the above two preparations.
4. Lipid-free rhodopsin: rhodopsin preparation obtained after lipid removal by affinity chromatography in detergent solution, irrespective as to whether the detergent is removed or not.

6.2.2. Acetylation of photoreceptor membrane components

Rod outer segment membranes, washed with distilled water, are suspended in half saturated sodium acetate in a concentration of 3 mg/ml (about 25 μM rhodopsin) and cooled to 0°C. Over a period of 10 minutes, five 40 μl-volumes of acetic anhydride are added under vigourous shaking to 20 ml of the suspension. Although this results in a decrease of pH 7.3 to 5.2, no rhodopsin is denaturated, since the 500 nm absorbance remains unchanged. The suspension is left standing for 1 hr at 20°C and is then centrifuged for 1 hr at 4°C (20,000 rpm, Sorvall RC2B, rotor SS34). Part of the preparation is twice washed with distilled water, lyophilized and used for the determination of free amino-groups (section 6.2.3). The rest is washed with 0.1 M phosphate buffer (pH 6.5), resuspended in this buffer and used for spectral measurements and photolytic product analysis.

Acetylation has also been performed in detergent solution. In that case 45 mg rod outer segment membranes (ca. 350 nmol of rhodopsin) are solubilized in 11 ml of an ice cold solution of 0.1 M dodecyltrimethylammonium bromide (DTAB) in half saturated sodium acetate. Over a period of 10 minutes, five 15 μl-volumes of acetic anhydride are added under agitation. The 500 nm absorbance is again not affected by this treatment. Subsequently, DTAB is removed by dialysis against 0.1 M phosphate buffer (pH 6.5) containing 1 mM DTE, as described in section 3.3.8. The resulting reconstituted membranes are collected by centrifugation at 75,000 x g (1 hr, 4°C). For further experimentation part of the preparation is washed with distilled water and lyophilized, while the rest is resuspended in 0.1 M phosphate buffer. More than 90% of rhodopsin is recovered in this procedure.

Control preparations are obtained by subjecting rod outer segment membranes to identical treatments as described above, omitting the addition of acetic anhydride. The control preparations of the second procedure are, in fact, rod outer segment membranes, reconstituted with their original lipids.

6.2.3. Determination of primary amino groups

The number of primary amino groups is determined by reaction with 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) as previously described (De Grip et al., 1973d; De Grip, 1974). When known weights of lyophilized preparations are solubilized in 2% aqueous Triton X-100 (40 - 200 μM amino groups), the total number of primary amino groups per molecule of rhodopsin can be calculated from the amino group determination and the ΔA_{500}/mg value of the lyophilized material (see section 3.3.3).

In order to discriminate between protein and lipid amino groups, an extraction procedure is employed. About 3 mg of a control or about 9 mg of an acetylated preparation are resuspended in 1.5 ml of 0.1 M KCl. The 500 nm absorbance and total phosphate content (after acid destruction, see section 3.3.6) are determined in aliquots. Subsequently 3.8 ml of chloroform-methanol (1.2, by volume) is added to 1 ml of the membrane suspension and the mixture is shaken for 15 minutes. Then 1.2 ml of $CHCl_3$ and 1.2 ml of 0.1 M KCl are added, and the mixture is shaken again. After phase separation, the organic lower phase is carefully removed and 4.7 ml of chloroform-methanol (2:1, by volume) is added to the aqueous residue. The organic layer is again removed after shaking and phase separation and the procedure is repeated once more. The combined organic extracts are evaporated to dryness. The resulting lipid film is solubilized in 3.5 ml of 2% aqueous Triton X-100 and this solution is used for the estimation of primary amino groups. From the phosphate determination after acid destruction of a sample of the Triton X-100 solution a recovery of > 95% is calculated. Therefore, the results of the primary amino group estimation can be expressed as the number of lipid-bound primary amino groups per rhodopsin molecule. The number of protein-bound primary amino groups per molecule of rhodopsin is found by substracting the number of lipid-bound primary amino groups from the total number of primary amino groups.

6.3. Results

6.3.1. Wavelength of visible absorbance peak

All rhodopsin preparations discussed in this chapter exhibit the same maximal absorbance wavelength of 498 nm. This is found by determining the absorbance difference spectrum before and after illumination in the presence of 50 mM NH_2OH. Therefore, we may conclude that the chromophoric centre of rhodopsin is not affected by the treatments to which rhodopsin has been subjected.

6.3.2. Effect of acetylation on the photolytic behaviour of rhodopsin

Upon treatment of native rod outer segment membranes with acetic anhydride, the total amount of primary amino groups per molecule of rhodopsin decreases from 58.4 to 5.9 (table 6.1). Sepa-

Table 6.1

EFFECT OF ACETYLATION ON PRIMARY AMINO GROUP CONTENT IN PHOTORECEPTOR MEMBRANES

photoreceptor membrane preparation	Number of NH_2-groups per molecule of rhodopsin*		
	total	lipid-bound	protein-bound
untreated	58.4	39.6	18.8
acetylated	5.9	< 0.6	5.3 - 5.9
reconstituted	58.7	—	—
acetylated in DTAB and reconstituted	2.3	—	—

*including the amino group binding the chromophore in rhodopsin

rate estimation of the lipid-bound amino groups shows that almost all lipid amino groups have been acetylated (detection limit is 0.6 NH_2-group per rhodopsin molecule). Thus about 13 out of 19 protein-bound amino groups per rhodopsin molecule have been acetylated.

Acetylation has also been carried out with rod outer segment

membranes solubilized in 100 mM DTAB. In order to reassemble the membrane constituents, the detergent is subsequently removed by dialysis (see section 6.2.2). Control preparations, where addition of acetic anhydride is omitted will be referred to as reconstituted rod outer segment membranes. The almost identical value of total amino groups per molecule of rhodopsin in native and reconstituted rod outer segment membranes shows that the amino group bearing phospholipids are completely recovered in the reconstitution procedure (table 6.1). After addition of acetic anhydride to DTAB solubilized photoreceptor membranes and subsequent detergent dialysis, it is found that only 2.3 amino group per rhodopsin molecule remain unmodified. Rhodopsin remains spectrally completely intact during all these manipulations, including acetylation. Since the amino groups are determined under denaturing conditions, one of the detected residual amino groups derives from the chromophore binding ε-amino group of a lysine residue (cf. De Grip et al., 1973d). Thus, after acetylation of rod outer segment membranes in DTAB solution, only 1.3 original primary amino group per rhodopsin molecule is left. This group is probably located on the protein in view of the relative ease by which lipid-bound amino groups are acetylated (see table 6.1),which has also been observed upon amino group modification by other agents (De Grip, 1974).

The photolytic behaviour of control preparations for the two acetylation procedures (native and reconstituted rod outer segment membranes) is very similar. Upon illumination of reconstituted rod outer segment membrane suspensions metarhodopsin II (λ_{max} = 380 nm) is the first detectable spectral intermediate*. Its decay to metarhodopsin III exhibits first order kinetics. Other characteristics, like difference spectra, total absorbance loss at 380 nm

*First detectable photoproduct (or spectral intermediate). The first photoproduct resolved in our spectrophotometric measurements. It implies that the concentration of that photoproduct is maximal within 10 sec. Earlier intermediates are not detected since their decay is complete within this period of time (see fig. 1.8).

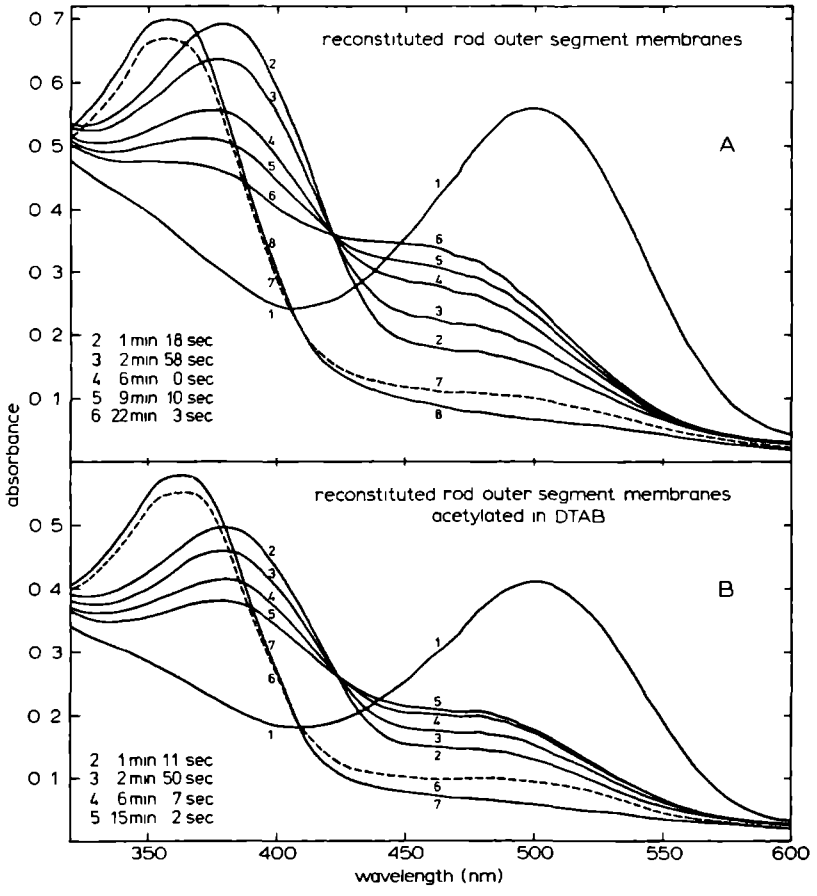


Fig. 6.1. Spectral recordings of the photolysis of control (A) and acetylated (B) reconstituted rod outer segment membranes. The preparations are obtained as described in section 6.2.2. Photolysis occurs in 0.1 M phosphate (pH 6.5) at 25°C. In both figures spectrum 1 is recorded before illumination. Spectra 2 to 6 in A and 2 to 5 in B have been recorded starting at the indicated times after the onset of illumination (30 sec behind OG_{530} filter, see section 5.2.1). Several intermediate spectra have been omitted for the sake of clarity. Spectrum 6 in A and 5 in B are almost time-invariant, which implies that the transition is about complete by then. The dotted spectra are obtained after completion of the transition and subsequent addition of NH_2OH (50 mM), and thus represent residual rhodopsin and retinylidene oxime. The last spectrum is obtained after exhaustive illumination in the presence of NH_2OH (no rhodopsin remains). All spectra are recorded at a scanning-speed of 6 nm/sec, starting at 600 nm. For comparison with the spectral transition of native rod outer segment membranes, see fig. 5.3.

and gain at 455 nm as well as the isosbestic point (417 nm), are also identical to those found in native rod outer segment membrane suspensions (compare fig. 5.3 and fig. 6.1.A). However, the transition rate is always increased, maximally by a factor of about 1.5, with respect to that found in native membrane suspensions (see chapter 5).

Both types of acetylated rod outer segment membranes exhibit exactly the same photolytic behaviour, whether the modification is performed by addition of acetic anhydride to a suspension or to a DTAB solution. There are two clear differences as compared to the photolytic behaviour of native rod outer segment membranes. First, the transition rate of metarhodopsin II to III is increased by a factor of maximally 2. Secondly, the fraction of metarhodopsin II that decays to metarhodopsin III is lowered, since the total absorbance loss at 380 nm during the transition is some 0.7 to 0.85 times that of control preparations, and the same is found for the gain at 455 nm (compare fig. 6.1.B).

When the metarhodopsin II to III transition is complete, the photoproduct composition of the control reconstituted rod outer segment membranes is not appreciably altered compared to that of native membranes (table 6.2). This confirms the spectral observa-

Table 6.2

EFFECT OF ACETYLATION IN DTAB ON COMPOSITION OF PHOTOPRODUCTS RESULTING FROM METARHODOPSIN II DECAY

photoreceptor membrane preparation	free retinal-dehyde %	lipid-bound retinylidene imine %	protein-bound retinylidene imine %
reconstituted	33.2	14.0	52.8
acetylated in DTAB and reconstituted	45.3	0	54.7
untreated*	33.8	15.2	51.0

Photoproduct composition is expressed as percent of total photoproduct concentration.
*data from table 5.2.

tions. Apparently mere reconstitution of rhodopsin into membranes with the original lipid composition of the photoreceptor membrane has hardly any effect on the photolytic behaviour of rhodopsin.

A clear effect is seen after acetylation. Lipid-bound retinylidene-imine is no longer a product of metarhodopsin II decay (table 6.2). This is obviously due to the complete blocking of phospholipid amino groups, so that the fraction of retinaldehyde which is normally bound to phosphatidylethanolamine now adds to the free retinaldehyde fraction, while the protein-bound fraction of photoproducts remains about the same as in the controls (see table 6.2).

6.3.3. Effect of phospholipase C treatment

Removal of the polar head groups from phospholipids in rod outer segment membranes is achieved by phospholipase C treatment (see section 3.3.10). This treatment does not appreciably affect the photolytic behaviour of rhodopsin as long as less than about 40% of the polar head groups have been removed. At higher levels of phospholipid hydrolysis, the photolytic behaviour of rhodopsin becomes more complex (compare fig. 6.2.A). A new intermediate appears and beyond 80% hydrolysis this is the only first detectable photoproduct (see fig. 6.2.C). The new intermediate exhibits maximal absorbance at about 480 nm (see fig. 6.2.D) and is very likely metarhodopsin I, which absorbs maximally at this wavelength (cf. Matthews et al., 1963). This view is supported by the following observation. Addition of detergent to photolyzed suspensions of phospholipase C treated membranes (about 90% hydrolysis), which contain substantial amounts of the 480 nm pigment, restores the appearance of the later spectral intermediates (metarhodopsin II and III and retinaldehyde) at least qualitatively. The photolysis pattern is then the same as that of native rod outer segment membranes illuminated in the same detergent. It indicates that the 480 nm pigment is on the normal route of the thermal decay of photolyzed rhodopsin, and that it preceeds the metarhodopsin II stage, which are both characteristics of metarhodopsin I. In view of the concomitant

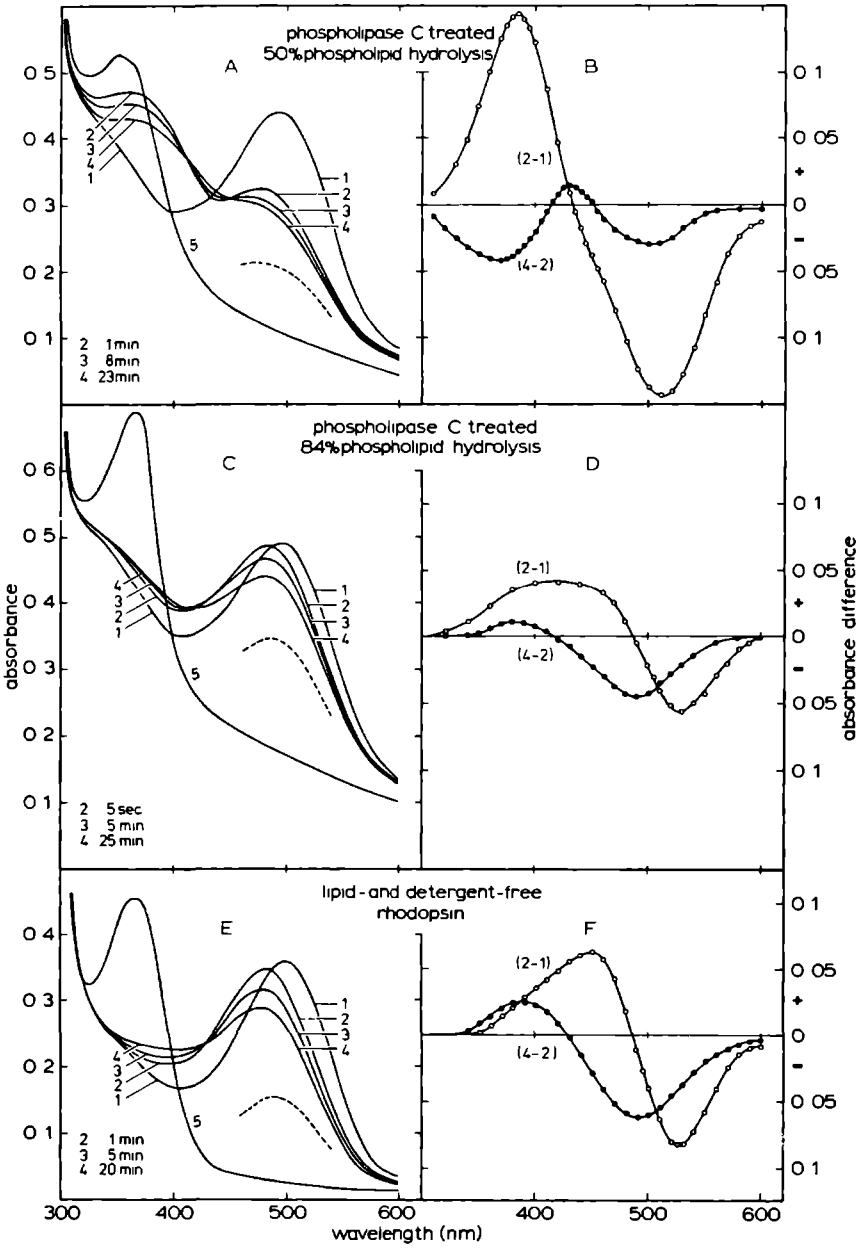

phospholipase C treated
50% phospholipid hydrolysis
A
B
(2-1)
(4-2)
2 1min
3 8min
4 23min
phospholipase C treated
84% phospholipid hydrolysis
C
D
2 5sec
3 5min
4 25min
lipid-and detergent-free
rhodopsin
E
F
2 1min
3 5min
4 20min
absorbance
absorbance difference
wavelength (nm)

Fig. 6.2. Spectral changes after illumination of phospholipase C treated rod outer segment membranes (A. 50% and B. 84% phospholipid hydrolysis) and of lipid- and detergent-free rhodopsin (E) with the absorbance difference spectra (B, D and F, respectively). Fig. A, C and E: spectrum 1 is recorded before illumination. Spectra 2, 3 and 4 are taken at the indicated times after the onset of illumination through an OG_{530} filter (30 sec by a tungsten source for A and E and light flash for C; compare section 5.2.1). Several intermediate spectra have been omitted for the sake of clarity. Spectrum 4 is almost time-invariant, which implies that the transition is complete. The dotted spectra, obtained after completion of the transitions and addition of 50 mM NH_2OH, derive from residual rhodopsin. After exhaustive illumination spectrum 5 is recorded, where no rhodopsin is left. All spectra are recorded at a scanning-speed of 6 nm/sec starting at 600 nm. The spectra are redrawn from original recordings, shortening the scale of the wavelengths. Fig. B, D and F: difference spectra between spectra 2 and 1 (open circles) and 4 and 2 (black dots) of figures A, C and E, respectively.

appearance of metarhodopsin I and metarhodopsin II in preparations with a phospholipid hydrolysis between about 40 and 80%, two types of rhodopsin obviously exist in these preparations. One type yields metarhodopsin II and the other gives metarhodopsin I as the first detectable photolytic product of rhodopsin. Going from 40 to 80% hydrolysis a gradual shift of rhodopsin from the first type to the second seems to occur. Beyond 80% hydrolysis metarhodopsin I is the only first detectable photoproduct. Apparently the metarhodopsin I decay of all molecules is greatly slowed down under these conditions, the half-life time of metarnodopsin I being between 5 and 10 minutes (few seconds in native preparations). Whether metarhodopsin II is still formed during this slow decay is not clear, since a tentative estimation shows that free retinaldehyde is a substantial decay-product.

6.3.4. Photolytic behaviour of lipid- and detergent-free rhodopsin

Suspensions of lipid- and detergent-free rhodopsin, obtained by affinity chromatography (see section 3.3.7) and detergent dialysis (see section 3.3.8), exhibit the same photolytic pattern as observed in phospholipase C treated membranes in which about 90% of the phospholipids is hydrolyzed (compare fig. 6.2.C and fig. 6.2.E).

Here again the first detectable photoproduct has its maximal absorbance at about 480 nm (see fig. 6.2.F). For the same reasons as given for the phospholipase C treated preparation (see previous section) this is very likely to be metarhodopsin I. The pigment again gradually decays with a half-life time of 5 to 10 minutes. The absorbance gain at 380 nm is substantially less than the absorbance loss at 480 nm, which is also found in the phospholipase C treated preparations (about 90% hydrolysis, compare fig. 6.2.D). Since there is an isosbestic point at 425 nm, this is probably not caused by trivial loss of chromophore, e.g. by oxidation. In addition, the chromophore absorbance is regained at 365 nm by treatment with hydroxylamine, which converts all photoproducts to retinylidene oxime (λ_{max} = 365 nm; see fig. 6.2.E, curve 5).

Although the photolytic behaviour of lipid- and detergent-free rhodopsin and of rod outer segment membranes exhaustively treated with phospholipase C is very similar, the physical appearance of the two preparations is quite different. Phospholipase C treatment causes an increase in light scattering as may be seen from the high absorbance at about 600 nm and tne rather strong increase in basic absorbance going from 600 to 450 nm in curves 5 of figs.6.2.A and C. These suspensions are rather unstable and therefore, the addition of 30% glycerol is often required. This does not affect the photolytic behaviour itself. The absence of a real isosbestic point during metarhodopsin I decay in preparations exhaustively treated with photpholipase C (see fig. 6.2.C) may be due to this instability (fairly rapid sedimentation). The instability is probably caused by the increased content of neutral lipids (diglycerides) at the expense of amphipathic lipids. None of these problems are encountered in suspensions of lipid- and detergent-free rhodopsin, which are rather transparent (cf. low absorbance and slight increase of absorbance going from 600 to 450 nm in fig. 6.2.E, curve 5).

Table 6.3

RESTORATION OF APPEARANCE AND DECAY OF METARHODOPSIN II

lipid[a]	RECONSTITUTION by the detergent dialysis procedure		SIMPLE ADDITION of lipids	
	PL-ase C treated membranes (± 90% hydrol.)	lipid- and detergent-free rhodopsin[d]	PL-ase C treated membranes (± 90% hydrol.)	lipid- and detergent-free rhodopsin
egg phosphatidylcholine	+	+	-	-
dioleoylphosphatidylcholine	+	+	-	-
didecanoylphosphatidylcholine	+	+	±	±
egg phosphatidylethanolamine	+	+	-	-
bovine phosphatidylserine[b]	+	+	-	-
phospholipid mixtures[c]	+	+	n.d.	-
phosphatidic acid	n.d.	+	n.d.	-
monogalactosyldiglyceride	n.d.	+	n.d.	-
rod outer segment lipids	+	+	±	-
1-mono-oleoylglyceride	-	-	-	-
1,2-di-oleoylglyceride	-	-	-	-
tri-oleoylglyceride	-	-	-	-

Measurements are carried out at 25°C in 0.1 M phosphate (pH 6.5). + = restoration, - = no restoration, ± = restoration occasionally observed, n.d. = not determined. a: lipid/rhodopsin molar ratios between 60 and 165. b: from spinal cord. c: Mixtures of egg phosphatidylcholine and bovine phosphatidylserine (molar ratio 90:10 and 50:50) as well as of egg phosphatidylcholine, egg phosphatidylethanolamine and bovine phosphatidylserine (molar ratio 70:15:15 and 45:45:10). d: the same results are obtained whether of not the pure rhodopsin is obtained in a detergent-free state, prior to the detergent dialysis procedure.

6.3.5. Recombination of delipidated rhodopsin with lipids by means of a detergent dialysis procedure

Assembly of rhodopsin with various lipids is achieved by solubilizing these substances in dodecyltrimethylammonium bromide (DTAB) and subsequent removal of this detergent by dialysis (see section 3.3.8). Employing a series of lipids, a strong parallelism is found between the photolytic behaviour of lipid-rhodopsin recombinants for which the rnodopsin source is either lipid-free rhodopsin (detergent-free or not) or rhodopsin from rod outer segment membranes exhaustively treated with phospholipase C (compare table 6.3).

When neutral lipids (oleoylglycerides) are combined with rhodopsin, the metarhodopsin I to II transition is largely blocked. A photolytic pattern is obtained which is identical to that of photoreceptor membranes exhaustively treated with phospholipase C (see section 6.3.3).

When rhodopsin is recombined with phospholipids, illumination of the reconstituted preparations leads to the immediate and exclusive appearance of metarhodopsin II, which (partially) decays to metarhodopsin III. Apparently the metarhodopsin I to II transition is no longer blocked and can, at least qualitatively , proceed normally. This does not depend on the nature of the polar head groups of the phospholipids. Even the strongly negative phosphatidic acid is suitable. The presence of a phosphate residue in the head group is also not required, since with monogalactosyldiglyceride similar results are obtained. All preparations reconstituted with amphipathic lipids exhibit first order kinetics of the metarhodopsin II to III transition. However, tne rates of transition is always increased, maximally by a factor of two as compared to that found in native rod outer segment membrane suspensions. The isosbestic point of the transition remains at about 417 nm, and the absorbance maximum of metarhodopsin III (455 nm) does not seem to be influenced by the nature of the polar head groups. No obvious effect of chain-length of the fatty acid residues is observed, since didecanoyl-($C_{10:0}$)- and

dioleoyl-($C_{18:1}$)-phosphatidylcholine show exactly the same photolytic pattern. Lipid/rhodopsin molar ratios of 60 to 165 appear to give the same results for any specific lipid or lipid mixture.

There is, however, a noticeable influence of the nature of the polar head groups on the quantitative formation of metarhodopsin III From the total absorbance loss at 380 nm and the gain at 455 nm between zero and infinite time of the transition, we estimate that 15 to 30% less metarhodopsin III is formed when phosphatidylethanolamine is absent in the reconstituted preparations. The spectral transitions are then the same as those observed for

Table 6.4

DEPENDENCE OF PHOTOPRODUCT COMPOSITION AFTER METARHODOPSIN II DECAY ON POLAR HEAD GROUP COMPOSITION OF PHOSPHOLIPIDS

preparation	free retinal-dehyde %	lipid-bound retinylidene imine %	protein-bound retinylidene imine %
lipid-free rhodopsin reconstituted with			
dioleoylphosphatidyl-choline*	44.0	0	56.0
egg phosphatidyl-ethanolamine*	9.0	34.3	56.7
bovine phosphatidyl-serine*	47.5	0	52.5
reference values from table 5.2 and 6.2.			
rod outer segment membranes	33.8	15.2	51 0
reconstituted rod outer segment membranes	33.2	14.0	52.8
reconstituted rod outer segment membranes acetylated in DTAB	45.3	0	54.7

Photoproduct composition is expressed as percent of total photoproduct concentration.
*Phospholipid/rhodopsin molar ratio is 66.

acetylated preparations (see section 6.3.2). Photoproduct analysis after completion of the metarhodopsin III formation in reconstituted membranes of lipid-free rhodopsin and pure dioleoylphosphatidylcholine or pure phosphatidylserine confirms that this effect is largely due to the formation of additional free retinaldehyde instead of lipid-bound retinylidene-imine (see table 6.4). An opposite effect is observed when phosphatidylethanolamine is the exclusive phospholipid in reconstituted membranes. Here, an increase of retinylidene-phosphatidylethanolamine at the expense of free retinaldehyde is observed at the end of the metarhodopsin II to III transition (see table 6.4), while the absorbance loss (at 380 nm) and gain (at 455 nm) are increased by 10 to 20% as compared to that found in native membranes. However, the protein-bound photoproduct fraction remains unaffected, whatever the nature of the polar head groups of phospholipids may be (table 6.4).

6.3.6 Mere addition of lipids to delipidated rhodopsin preparations

In general mere addition of lipids (see section 3.8.9) to suspensions of membranes exhaustively treated with phospholipase C or of lipid- and detergent-free rhodopsin does not result in alteration of the photolytic behaviour of these preparations (table 6.3). The metarhodopsin I decay remains strongly hampered. There are, however, occasional exceptions. Some phospholipase C treated rod outer segment membranes (about 90% phospholipid hydrolysis) show a reasonably normal photolytic pattern (appearance and decay of metarhodopsin II), when lipids extracted from rod outer segment membranes, or didecanoylphosphatidylcholine are added to the suspension. The addition of the latter compound to lipid- and detergent-free rhodopsin suspensions shows in some cases a recovery of the photolytic pattern as is found for the reconstituted preparations of these substances obtained by the dialysis procedure. The other lipids which we have tested do not show such effects (table 6.3). Addition of 20 mM $MgCl_2$ or of 30% glycerol does not change this, not even after sonication

(3 times, 2 sec., 0°C).

6.4. Discussion

6.4.1. The decay-routes of metarhodopsin II

In rod outer segment membrane suspensions metarhodopsin II exhibits three possible decay-routes, as concluded from the ultimate formation of three different stable products: free retinaldehyde, retinylidene-phosphatidylethanolimine and retinylidene-opsin (see chapter 5). Photoproduct analysis after metarhodopsin II decay of rhodopsin reconstituted with either phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine, shows that always about the same fraction of ultimate photoproducts is protein-bound (see table 6.4). The same fraction is also found when rod outer segment membranes are acetylated in DTAB and reassembled by detergent dialysis (see table 6 4)

The ratio of free retinaldehyde to retinylidene-phosphatidylethanolimine seems to be related to the fraction of phosphatidylethanolamine in the total phospholipid pool. When rhodopsin is reassembled with exclusively dioleoylphosphatidylcholine of phosphatidylserine by the dialysis procedure, metarhodopsin II decay does not result in a lipid-bound retinaldenyde fraction, as is also found in acetylated membranes (see table 6.4). Phosphatidylserine is apparently not a good acceptor for retinaldehyde during metarhodopsin II decay and phosphatidylcholine does not even have a primary amino group. In native and reconstituted rod outer segment membranes phosphatidylethanolamine comprises about 40% of the total phospholipid pool (compare table 3.2) and in these preparations about 15% of the photoproducts of metarhodopsin II consist of retinylidene--phosphatidylethanolimine. This percentage is increased to about 35%, when phosphatidylethanolamine is the exclusive phospholipid of the reconstituted membranes (see table 6.4).

In metarhodopsin II retinaldehyde is completely bound to opsin

(see section 5.3.2) and thus the lipid-bound photoproducts show that during metarhodopsin II decay transiminization occurs and that this process depends on the availability of primary amino groups of phosphatidylethanolamine. In all preparations studied in this chapter the rate of metarhodopsin II decay is increased maximally by a factor two, as compared to that in native rod outer segment membranes. This observation is not easily explained. The effect is not necessarily due to the presence of residual detergent, since rod outer segment membranes treated with acetic anhydride show the same increase. It has apparently no implications for the formation of photoproducts during metarhodopsin II decay, since reconstituted rod outer segment membranes exhibit similar increases in transition rate, while the ultimate photoproduct composition is identical to that found for native photoreceptor membranes (see table 6.4).

6.4.2. The nature of metarhodopsin III

Thus we have established that metarhodopsin II decay always gives a constant fraction of opsin-bound retinaldehyde and that the presence of phosphatidylethanolamine determines the fate of the other retinaldehyde molecules. It now seems possible to correlate the spectral analysis with the results of photoproduct analysis, which was not possible for native rod outer segment membranes (see discussion in chapter 5). On the basis of the present observations we may arrive at the following deductions concerning the nature and formation of metarhodopsin III in suspensions of photoreceptor membranes:

1. METARHODOPSIN III IS NOT A SINGLE COMPONENT

In the absence of phosphatidylethanolamine 15 to 30% less metarhodopsin III is formed than in native or reconstituted rod outer segment membranes. A similar difference is observed after acetylation. This observation shows that most of the metarhodopsin III absorbance derives from the constant fraction of opsin-bound retinylidene imine. Since this fraction together with that of retinylidene-phosphatidylimine comprises about 66% of the total photoproducts in native pho-

toreceptor membranes (table 6.4), elimination of this imine would lead to a theoretical loss of metarhodopsin III absorbance of about 22%, assuming identical spectral characteristics for both the lipid- and protein-bound retinylidene-imines. This theoretical decrease is within the range of absorbance decreases actually observed (15 to 30%). In addition an increase in metarhodopsin III absorbance is observed when phosphatidylethanolamine is the only phospholipid present. Therefore, we may conclude that the metarhodopsin III absorbance derives from the fractional spectral contributions of two compounds: lipid- and protein-bound retinylidene-imine. In view of the isosbestic point observed during metarhodopsin III formation, these two compounds appear to be spectrally indistinguishable.

2. THE MOLAR ABSORBANCE OF METARHODOPSIN III AT 455 nm IS 27,000

In native rod outer segment membrane suspensions the total absorbance loss at 380 nm during the metarhodopsin II to III transition is 1.5 times the gain at 455 nm (see chapter 5). The same factor is found in reconstituted preparations, whether or not phosphatidylethanolamine is present. This apparent absorbance loss can, in view of the previous conclusion, only be explained by assuming that the molar absorbance of metarhodopsin III at 455 nm is about 1.5 times less than the molar absorbance of metarhodopsin II at 380 nm. In digitonin the molar absorbances of these two intermediates are found to be nearly equal (cf. Matthews et al., 1963). The discrepancy with our observations must be due to the presence of detergent.

We have found that upon photolysis the total absorbance loss of rhodopsin at 500 nm is equal to the total absorbance gain of metarhodopsin II at 380 nm (see fig. 5.4.A). Therefore, we may conclude that the molar absorbances of rhodopsin and metarhodopsin II at their respective absorption maxima are identical. This is also found in digitonin solutions of bovine photoreceptor membranes (cf. Matthews et al., 1963). On the basis of a molar absorbance of 40,500 for rhodopsin (Daemen et al., 1972), we thus may conclude that in suspensions of photoreceptor membranes the molar absorbance of metarhodopsin III at 455 nm is about 27,000.

3. THE SPECTRAL COMPONENT METARHODOPSIN III IS NOT THE SUM OF THE ABSORBANCES OF RANDOMLY DISTRIBUTED RETINYLIDENE-IMINES

Indeed, during the transition of metarhodopsin II to III retinaldehyde transiminizes from the original chromophore binding site to other primary amino groups. This is shown by the observation that part of the retinaldehyde can become bound to phosphatidylethanolamine. For the protein-bound component of metarhodopsin III this is shown by regeneration experiments after reductive fixation of all retinylidene-imines (cf. Rotmans et al., 1974) and also by the observation that metarhodopsin II, but not metarhodopsin III, is formed when all primary amino groups of the opsin molecule, except for the chromophore binding site, are blocked by modifying agents (cf. De Grip, 1974). However, since the fraction of lipid-bound retinylidene-imine is much smaller than the fraction of phospholipid amino groups (see table 6.1), a real random binding of retinaldehyde to all available primary amino groups does not occur. In addition, proteolytic treatment of rod outer segment membranes shows that substantial parts of the rhodopsin molecule, including lysine residues (cf. Trayhurn et al., 1974b, Saari, 1974), are not involved in the metarhodopsin III formation, since their removal has no detectable effect on the photolytic behaviour (see section 4.3.1).

4. ONE SPECIFIC PROTEIN BOUND AMINO GROUP ACTS AS ACCEPTOR FOR RETINALDEHYDE IN THE TRANSIMINIZATION PROCESS

This conclusion is based on the observation that only about one primary amino group is left after acetylation of DTAB solubilized rod outer segment membranes, while the fraction of metarhodopsin III which has the protein bound to opsin still occurs after removal of the detergent (see section 6.3.1). Although there is no direct experimental evidence that the only residual primary amino group is the same in all rhodopsin molecules, the preceding and following arguments make this likely.

5. THE CHROMOPHORE UNDERGOES ONLY A SLIGHT DISPLACEMENT DURING METARHODOPSIN III FORMATION

The absorbance maximum at 455 nm of metarhodopsin III cannot simply be explained by assuming that it derives from a normal protonated aliphatic retinylidene-imine, since such a compound exhibits maximal absorbance at about 440 nm, in solution (see section 5.2.2) as well as in suspension. Protonated retinylidene-phosphatidylethanolimine is formed upon addition of all-trans retinaldehyde to non-illuminated rod outer segment membrane suspensions, but even under these conditions its absorbance maximum never exceeds 445 nm (cf. Shichi and Somers, 1974). The bathochromic shifts of absorbance maxima exhibited by rhodopsin and its photolytic intermediates are probably due to specific interactions between the chromophore and the surrounding protein parts (see section 1.4.3). If we assume that similar interactions are responsible for the bathochromic shift in metarhodopsin III, it follows that in this spectral component the polyene-chain of retinaldehyde, either bound to opsin or to phosphatidylethanolamine, must have a specific location on the protein. The absorbance maximum of metarhodopsin II (380 nm) shows about the same red shift of 15 nm, compared to normal unprotonated aliphatic retinylidene-imines (λ_{max} = 365), as in metarhodopsin III. Hence, the retinaldehyde moiety in metarhodopsin III is probably in about the same position as in metarhodopsin II. This conclusion is supported by linear dichroism measurements, which show that in metarhodopsin III the chromophore orientation is not much different from that in rhodopsin and its photolytic intermediates (cf. Harosi, 1975; see also section 2.2.8). Therefore, during the transiminization process, accompanying the metarhodopsin II to III transition, only a relatively small displacement of the aldehyde-end of retinaldehyde from the original chromophore-binding to a nearby primary amino group can occur.

In rod outer segment membranes there is also a retinylidene--phosphatidylethanolimine fraction contributing to metarhodopsin III. In view of the slight displacement of this chromophore the amino group of phosphatidylethanolamine probably must approach the

original binding site as closely as the specific protein-bound amino group to accept the chromophore from the pigment in the metarhodopsin II state. Similarly a water molecule possibly can approach the original binding site to accept the chromophore from the pigment in the metarhodopsin II state, resulting in free retinaldehyde.

6. THE PROTONATION OF THE PROTEIN-BOUND RETINYLIDENE-IMINE IS NOT CAUSED BY A PHOSPHATE RESIDUE OF PHOSPHOLIPIDS

Since metarhodopsin III is still formed when monogalactosyldiglyceride is the only lipid in a rhodopsin-lipid recombinant, the protonation cannot derive from a phosphate residue of phospholipids. This conclusion is, of course, only correct, if a protonated retinylidene-imine does indeed exist in the metarhodopsin III state. The presence of a retinylidene-imine is beyond doubt, but our product analysis procedures do not distinguish between the protonated and unprotonated forms. However, in view of the absorbance maximum of metarhodopsin III (455 nm) the protonated form of retinylidene--imine is highly probable.

Metarhodopsin II is not a stable compound (at 25°C), possibly due to strain in the polyene-chain of retinaldehyde. Three different groups may act as an acceptor for the chromophore water, a phosphatidylethanolamine amino group or a specific protein amino group. The ratio of photoproducts may be determined by the average time that - in the metarhodopsin II state - these groups are in the optimal position for accepting the chromophore. Detailed kinetic studies, especially in dependence on temperature and pH, will, however, be needed to draw a more complete picture of the precise mechanism of the metarhodopsin II decay, including the possible existence of equilibria between the ultimate photoproducts and of fast consecutivity in their formation.

6.4.3. Influence of the micro-environment on the photolytic behaviour of rhodopsin

A clear effect on the photolytic behaviour of rhodopsin in photoreceptor membranes is exerted by phospholipase C. The action of this lipolytic enzyme reduces the amount of amphipathic lipids in the membrane. Apparently increased hydrolysis causes changes in a larger number of rhodopsin molecules. The metarhodopsin I decay of all molecules is strongly hampered, when more than 80% of the phospholipids are hydrolyzed, which is also observed in suspensions of lipid- and detergent-free rhodopsin.

Mere addition of amphipathic lipids to these delipidated rhodopsin preparations, without the intermediate use of detergent, does not restore the normal photolytic pattern. However, it appears that the damage caused by the removal of phospholipids can be restored by reassembly of the delipidated rhodopsin preparations with amphipathic lipids, provided that this is done by solubilization of rhodopsin and lipids in a detergent (DTAB), which is subsequently removed by dialysis. Neutral lipids are ineffective. The nature of the polar head groups of the amphipathic lipids does not seem to be critical. Phospholipids are not essential, since monogalactosyldiglyceride restores the photolytic behaviour of rhodopsin as well. It is known that the detergent dialysis procedure for reassembly of rhodopsin with amphipathic lipids leads to a reincorporation of the pigment molecule in lipid bilayers (Chen and Hubbell, 1973; Hubbell, 1975). Apparently rhodopsin must be surrounded by a lipid bilayer in order to show an qualitatively normal photolytic behaviour.

However, the last stage of the photolytic pattern of rhodopsin molecules in native or in reconstituted membranes may be quantitatively different. Metarhodopsin II always decays to metarhodopsin III and free retinaldehyde, but the ratio between these photoproducts is partially dependent on the nature of polar head groups of amphipathic lipids (compare section 6.4.1). Nevertheless, the fraction of metarhodopsin II decay-products that remains bound to the protein is always constant and represents the major part of meta-

rhodopsin III. Therefore,the quantitative differences between the photolytic behaviour of rhodopsin in native and reconstituted membranes are not an effect of changes in the protein molecule. Thus we may arrive at the following important conclusion: In a lipid bilayer rhodopsin is, with regard to its photolytic behaviour, not influenced by the composition of the surrounding amphipathic lipids.

In the next chapter we shall extend these studies of the effects of micro-environmental changes on the properties of rhodopsin by using thermal stability and regeneration capacity as criteria for the condition of rhodopsin. In chapter 8 electronmicroscopic studies of photoreceptor membranes treated with phospholipase C are reported. In chapter 9 we shall combine the results of this chapter and the next two chapters in order to arrive at a more complete interpretation of the effects of the micro-environment on the structure and function of rhodopsin.

CHAPTER 7

INFLUENCES OF THE MICRO-ENVIRONMENT OF RHODOPSIN ON THE THERMAL STABILITY AND REGENERATION CAPACITY

7.1. Introduction

In this chapter we shall present further aspects of our studies of the effects on the properties of rhodopsin of changes in its micro-environment. Using the photolytic behaviour as the criterion we have seen (chapter 6) that rhodopsin is affected by partial or complete lipid removal, and that a complete restoration of the photolytic behaviour is obtained when rhodopsin is reassembled with amphipathic lipid by the intermediate use of detergent. Here we want to study whether similar phenomena are observed when applying thermal stability and regeneration capacity as parameters of the structure and function of rhodopsin.

7.2. Methods

7.2.1. Preparations

Rod outer segment membranes are isolated as described in section 3.3.1, and are washed with water. Phospholipase C treatment is performed as presented in section 3.3.10. The isolation of lipid- and detergent-free rhodopsin is accomplished according to the procedure of section 3.3.8. The recombination of rhodopsin with lipids is conducted by a detergent dialysis procedure described in the same section. Mere addition of lipids to rhodopsin preparations, without the intermediate use of detergent is performed as described in section 3.3.9. Acetylation of primary amino groups is achieved according to

the procedure presented in section 6.2.2. All preparations used in the present chapter are virtually the same as used in the previous chapter.

7.2.2. Determination of thermal stability

The thermal stability of rhodopsin is determined as described in section 3.3.5. It is expressed as the temperature at which half of the 500 nm absorbance is lost in ten minutes of incubation.

7.2.3. Determination of regeneration capacity

The regeneration capacity of a rhodopsin preparation is the percentage of the original rhodopsin molecules that, after illumination, can be reconverted into rhodopsin by treatment with 11-cis retinaldehyde. It is determined as described in section 3.3.4.

A complication arises with lipid- and detergent-free rhodopsin or phospholipase C treated membranes, where photolysis results in a rather stable 480 nm pigment (metarhodopsin I, see previous chapter). Although in metarhodopsin I retinaldehyde is still bound to the original binding site, regeneration with 11-cis retinaldehyde can occur from this intermediate. When NH_2OH is added during photolysis, the 480 nm intermediate is rapidly decomposed to retinylidene-oxime (λ_{max} = 365 nm) and excess NH_2OH can be removed by washing and centrifugation. The regeneration capacity of such a sample is the same as found by the standard procedure, in which the intermediate is not decomposed prior to addition of 11-cis retinaldehyde.

Rhodopsin regeneration is performed by incubating the preparation with excess retinaldehyde for 1.5 hr. In all cases the regeneration of rhodopsin is maximal at this time, since longer incubation does not give rise to a further increase of the rhodopsin content of the preparation.

7.3. Results

7.3.1. Effects on thermal stability

Table 7.1 shows the thermal stability of various rhodopsin preparations, expressed as the temperature at which the 500 nm decreases by 50% in 10 minutes.

Table 7.1

THERMAL STABILITY OF THE 500 nm ABSORBANCE OF RHODOPSIN PREPARATIONS

Preparation	$T_{50\%}$ ($^{\circ}C$)*
native rod outer segment membranes	70.1
rod outer segment membranes treated with phospholipase C (Ca. 90% hydrolysis)	58.9
lipid and detergent-free rhodopsin	52.8
reconstituted membranes of rhodopsin and egg phosphatidylcholine** (detergent dialysis procedure)	70.1

*Temperature at which 50% of the 500 nm absorbance is lost in 10 minutes in 0.1 M phosphate buffer (pH 6.5).

**Molar ratio rhodopsin: phosphatidylcholine = 1:66.

Treatment of rod outer segment membranes with phospholipase C (90% phospholipid hydrolysis) decreases the thermal stability of rhodopsin. Complete removal of all lipids by affinity chromatography and detergent dialysis, yielding lipid- and detergent-free rhodopsin, causes an even greater decrease in thermal stability. A complete recovery of the thermal stability is observed, when membranes are reconstituted from lipid-free rhodopsin and egg phosphatidylcholine by means of the detergent dialysis procedure. The thermal stability of rhodopsin in native rod outer segment membranes is found to be 70.1$^{\circ}C$, which value fairly well agrees with the value of 72.6$^{\circ}C$ reported by Hubbard (1958) for bovine rod outer segments.

7.3.2. Effects on regeneration capacity

The regeneration capacity of rhodopsin in native rod outer segment membranes is about 92% (see table 7.2). Addition of acetic

Table 7.2

REGENERATION CAPACITY OF RHODOPSIN PREPARATIONS

Preparation	regeneration % (± SD)	n	mol phospholipid per mol rhodopsin
Suspensions:			
native rod outer segment membranes	92 ± 2	7	60 - 70
rod outer segment membranes treated with phospholipase C (ca. 90% hydrolysis)	47 ± 6	5	< 9
reconstituted rod outer segment membranes acetylated in DTAB	84	1	67
lipid- and detergent-free rhodopsin	48 ± 7	4	< 0.3
reconstituted membranes of rhodopsin and various lipids* (detergent dialysis procedure)	85 ± 3	21	60 - 165
Solutions in 100 mM DTAB:			
all rhodopsin preparations	0		variable

*The same lipids are used as in table 6.3, including the glycolipid monogalactosyldiglyceride, but not didecanoylphosphatidylcholine and oleoyl glycerides. n = number of determinations.

anhydride to solutions of rod outer segment membranes in 100 mM DTAB results in acetylation of all but 1.3 primary amino groups (see section 6.3.2). This preparation, after removal of the detergent by dialysis, still exhibits a regeneration capacity of 84% (table 7.2). This figure is the same as found for the control reconstituted rod outer segment membranes, in which the addition of acetic anhydride

is omitted. Treatment of rod outer segment membranes with phospholipase C initially hardly affects the regeneration capacity, but from 40-50% of phospholipid hydrolysis on, a gradual decrease is observed, until ultimately at about 90% hydrolysis, a regeneration capacity of about 50% is found (see fig. 7.1, table 7.2). Lipid- and

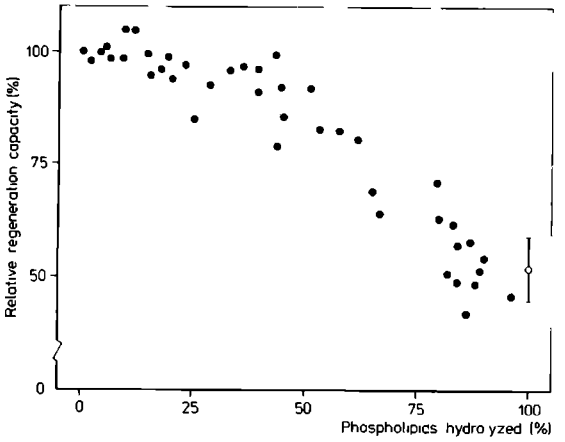


Fig. 7.1. Relative regeneration capacity of rod outer segment membrane preparations treated with phospholipase C as a function of the percentage phospholipid hydrolysis. The results of phospholipase C treatment of a number of preparations are combined in this figure. The regeneration capacity is espressed as percentage of the regeneration capacity of the corresponding untreated preparations, which exhibit an average regeneration capacith of 92% (see table 7.2). The open circle represents the regeneration capacity of lipid- and detergent-free rhodopsin suspensions (bars: + SD). It is expressed in percent of the average regeneration capacity of untreated rod outer segment membranes.

detergent-free rhodopsin also shows a lowered regeneration capacity of about 50% (table 7.2, see also Hong and Hubbell, 1973).

When either lipid- and detergent-free rhodopsin or phospholipase C treated rod outer segment membranes (about 90% phospholipid hydrolysis) are reassembled with amphipathic lipids by addition of detergent and lipids followed by detergent dialysis, almost complete restoration of the regeneration capacity is found (table 7.2). All the amphipathic lipids listed in table 6.3, show this result. Only

didecanoylphosphatidylcholine gives a slightly lower value (71% and 73%, n = 2). Reassembly with neutral lipids by means of the detergent dialysis procedure does not improve the regeneration capacity. Simple addition of lipids is also ineffective with respect to the regeneration capacity, except in those cases where the original photolytic sequence is restored.

7.4. Discussion

As judged by the effect on the thermal stability, rhodopsin suffers damage from reduction of the amount of amphipathic lipids caused by exhaustive phospholipase C treatment. Lipid- and detergent--free rhodopsin shows even a greater decrease in thermal stability. Complete recovery is observed after reconstitution of rhodopsin into a lipid bilayer of phosphatidylcholine.

Although the thermal stability of rhodopsin has not been studied for a series of reconstituted preparations, the present results strongly suggest that the thermal stability of rhodopsin depends on the presence of ampnipathic lipids, i.e. on the presence of a lipid bilayer surrounding rhodopsin. Indeed there is a difference in the thermal stability of a membrane preparation treated exhaustively with phospholipase C and that of lipid- and detergent-free rhodopsin, but in the former preparation there are always some residual phospholipids present and also relatively high concentrations of diglycerides, and this may cause that difference

The regeneration capacity is also affected by reducing the amount of amphipathic lipids. Both the membranes treated exhaustively with phospholipase C and lipid- and detergent-free rhodopsin show a decreased regeneration capacity compared to native rod outer segment membranes. A gradual decrease is observed when phospholipid hydrolysis is increased from 40 to 80%. A similar gradual alteration in dependence on the degree of phospholipid hydrolysis is observed when the photolytic behaviour of rhodopsin is used as criterion (see section 6.3).

There is a strong parallellism between the photolytic behaviour and the regeneration capacity of the two types of delipidated rhodopsin preparations with respect to the effects of their reassembly with lipids, either with or without the intermediate use of detergent. In fact table 6.3 also completely applies to the regeneration capacity when - stands for about 50% regeneration, + for more than 80% regeneration (except for reconstitution with didecanoylphosphatidylcholine, which gives about 72%), and ± for an occasionally observed restoration of the regeneration capacity to the same extent as is found after recombination by the detergent dialysis procedu e.

In order to obtain more information on the effects of phospholipase C treatment on the structural organization of the photoreceptor membrane, we have carried out electronmicroscopic studies, involving both sectioned and freeze-fractured preparations. The results of these studies are presented in the next chapter. An integrated discussion of the findings of chapter 6, 7 and 8 will be deferred to chapter 9.

CHAPTER 8

ELECTRON MICROSCOPY

8.1. Introduction: principle of freeze-fracturing and freeze-etching electronmicroscopy

8.1.1. Technique

The freeze-fracturing and freeze-etching techniques are two modes of a technique which aims at studying structural details of submicroscopic entities with a minimum of damage occurring during preparation of the sample. Essential feature of the method is fixation by means of very rapid freezing, so that formation of large ice-crystals which may disturb the architecture of the specimen is prevented (e.g. Costello and Gulik-Krzywicki, 1976). Freezing is generally performed by immersing the preparation in a cooling agent like liquid propane (-190°C) or melting Freon 22 ($CHClF_2$, -160°C). Freezing rates of 600 - 1000°C/sec are common, but rates of up to 100,000°C/sec may be achieved. Cryoprotective agents (like glycerol) are frequently added, since they lower the freezing point of tissue fluids, so that the time available for the growth of ice-crystals is shortened (smaller crystals). Chemical fixation of a biological preparation (e.g. by glutaraldehyde) may be carried out prior to freezing.

The frozen sample is mounted on a cooled stage (< -120°C in vacuum). By means of a rapid blow with a knife the specimen is cleaved so as to reveal a fresh, previously unexposed surface. This surface will show a relief of the structural details included in the object (see fig. 8.1). Since the fracture-face is not readily accessible to electronmicroscopic observation under these conditions, it has to be replicated. This is achieved by coating the surface with a thin layer of electron-dense material, mostly a noble metal

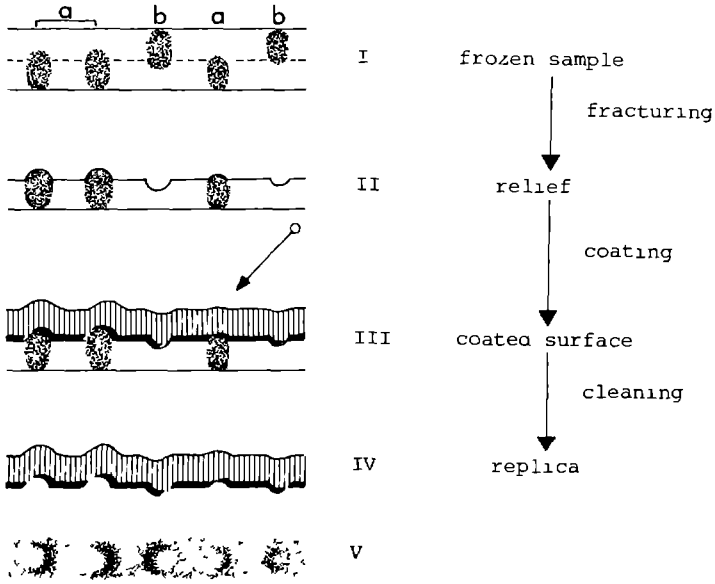


Fig. 8.1. Sequence of steps in the freeze-fracture technique. A description is given in the text. In III the arrow indicates the direction of coating by Pt/C (black areas). The carbon backing is evaporated onto the fracture-face in vertical direction. V: image obtained by electronmicroscopic observation perpendicular to the plane of fracturing, assuming spherical top of relief structures. Note that the actual shadows have a low electrondensity and thus have a light appearance in electronmicroscopic examination.

like platinum. This is evaporated onto the exterior of the fractured specimen by means of an electron gun under a specific angle from one direction. Due to the relief of the surface of the sample the structural details will then be resolved by shadow building (see fig. 8.1). Recently the feasibility of rotary shadowing has been shown (Margaritis et al., 1977). The best resolution is obtained, when the layer of noble metal is very thin. Since such a layer is fragile, it is usually reinforced by subsequent coating with a layer of carbon. At this stage, replication is completed and after removing of the sample (commonly by acid destruction), the clean replica can be examined in an electronmicroscope.

The freeze-etching technique is identical to freeze-fracturing, except that a short period for sublimation of ice from the frozen specimen is allowed between fracturing and replication. Sublimation is achieved by briefly increasing the temperature of the specimen between -110 and -100°C under very low pressure and generally in the absence of glycerol. By this treatment additional structures, which previously had been covered with ice, are revealed and the relief of the surface is enhanced (compare figs. 8.2 and 8.3).

8.1.2. Interpretation

In a lipid bilayer at low temperature the hydrophobic binding between the methyl-ends of the alkyl chains of the amphipathic lipids is relatively weak. This results in preferential fracturing along the plane of the endings of the alkyl chains, the so-called hydrophobic core of the bilayer (see fig. 2.1). Biological membranes contain in addition to a bilayer of amphipathic lipids, membrane proteins. When these proteins penetrate both halves of the lipid bilayer, they can go upon freeze-cleavage with either one of the two monolayers. Thus they produce relief in the fracture-face. Hills will occur on the surface to which the protein remains attached and they can be recognized as such in electronmicroscopic observation of the replica of that surface (fig. 8.1: a). Pits or holes are expected to occur, when the proteins are ripped out of the lipid bilayer (fig. 8.1: b)

There is no doubt that the particles, which are recognized as hills at the fracture-faces of biological membranes are related to intrinsic membrane proteins (cf. e.g. Pinto da Silva and Nicolson, 1974), but it has not yet been unambiguously assessed whether they derive from mere protein molecules or from protein-lipid complexes or even only from deformation of the lipid layer caused by underlying proteins. Holes expected from proteins ripped out of the replicated bilayer half are often not found (compare EF and PF face in fig. 8.6, which in principle are complementary to each other). This is not satisfactorily explained as yet. Therefore,

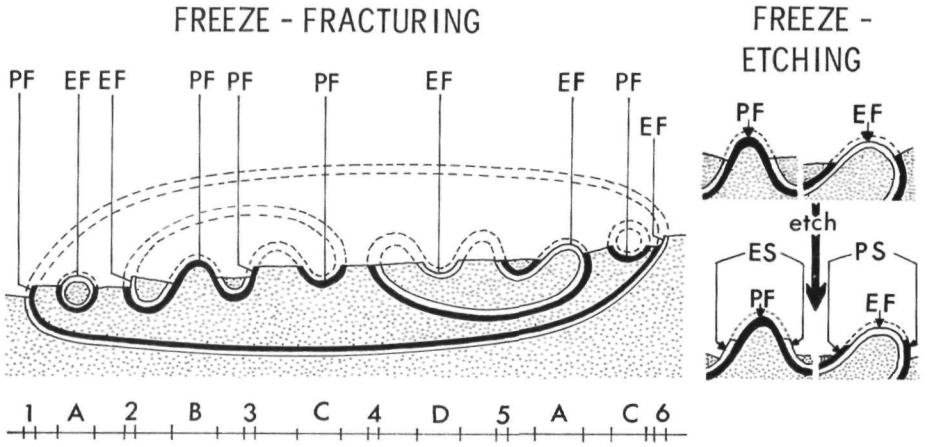


Fig. 8.2. Designation of surfaces observed after freeze-fracturing and freeze-etching. The membranes (lipid bilayers) are drawn in black and white. The black layer represents the protoplasmic half of the lipid bilayer. The white layer represents the exoplasmic half. Broken lines indicate structures which are torn away by freeze-cleavage. Dotted areas: ice. The exposed membrane surfaces are indicated by letters and figures at the bottom of the figure. Fracture-faces can be identified as protoplasmic (PF) or exoplasmic (EF) from concave or convex appearance, when they derive from spherical structures of single membrane orientation. In irregularly structured membrane vesicles discrimination is not possible on this basis. Convex EF faces (A) as well as convex PF faces (B) may be found. Also concave PF faces (C) as well as concave EF faces (D) may be obtained. Small fracture-faces are seen at 1, 2, 3 and 6. Cross-fracturing of a membrane is seen at 4 and 5. Most of these phenomena can be observed in fig. 8.6. On the right, freeze-etching is shown to disclose also the hydrophilic (outer) surfaces of membranes; protoplasmic surface: PS; exoplasmic surface: ES (see fig. 8.3 for an example). The designations PF, EF, PS and ES are in accordance with a nomenclature proposal by Branton et al. (1975).

freeze-fracture studies alone cannot show that the particles represent proteins that intrude into both halves of the lipid bilayer, let alone whether they span the entire width of the membrane. A critical discussion of this matter with respect to the photoreceptor membrane has been presented by Corless et al. (1976).

Nevertheless, since the particles are definitely related to the intrinsic membrane proteins, their distribution over the fracture face (and thus laterally in the membrane) is an important and

useful parameter for the study of protein-lipid relationships under various conditions (compare sections 2.2.3 and 2.2.6). Other parameters as particle size (estimated from the width of the shadow) and their density (number of particles per μ^2) can also be usefully employed.

Four different faces of biological membranes must be distinguished: two hydrophilic (outer) surfaces and two hydrophobic surfaces, viz. interfaces deriving from the interior of the membrane. In freeze-fractured specimens only the hydrophobic surfaces can be seen. The hydrophilic surfaces are disclosed only by etching (compare fig. 8.2). An example of a freeze-etched photoreceptor membrane is presented in fig. 8.3. Cell membranes possess one hydrophilic

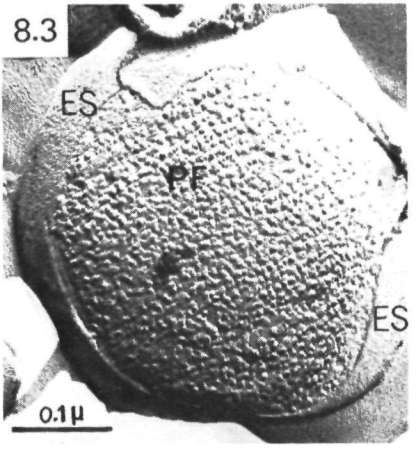


Fig. 8.3. Freeze-etched photoreceptor membrane (2 min. etching). The particle rich protoplasmic fracture-face (PF) is adjacent to the demasked smooth, hydrophilic exoplasmic surface (ES). For details of the technique, see section 8.1.1.

surface facing the extracellular fluid (exoplasmic, E) and one facing the intracellular fluid (protoplasmic, P). According to the nomenclature proposed by Branton et al. (1975) these surfaces are designated as ES and PS, respectively (see fig. 8.2). Such a discrimination also applies to closed membrane structures (organelles) floating within the intracellular fluid, as in the case of discal membranes in rod outer segments. The hydrophilic surface facing the cytoplasm (i.e. protoplasm) is designated as PS. The fluid interior of the organelles is denoted for the purpose of nomenclature conven-

tion as exoplasm and, therefore, the hydrophilic surface facing this "exoplasm" is denoted as ES. The hydrophobic fracture-face corresponding to the exoplasmic half of a membrane is coded as EF and that corresponding to the protoplasmic half as PF (see fig. 8.2).

Fracture-faces of spherical membrane vesicles are either convex or concave, and in a population of identical vesicles the respective faces can thereby be recognized and identified. The assessment of fractured faces and etched faces in irregular membrane structures cannot be achieved so readily (cf. fig. 8.2). Additional structural information is required, e.g. by protein labelling techniques or from specific repetitive structures, as is the case in rod outer segments. For rod outer segment membranes it is concluded that the intramembranous particles - which must derive from (monomers or oligomers of) rhodopsin - exhibit upon freeze-fracturing a great preference for the protoplasmic half of the membrane and thus the PF face is always found to be rich in particles (cf. Olive and Benedetti, 1974; Corless et al., 1976). The EF face is rather smooth and commonly no pits are revealed (see fig. 8.6). Such a preference of particles to remain in the protoplasmic half of membranes is frequently observed. The ratio between the particle densities at the PF and EF face is called the partition coefficient (Satir and Satir, 1974). This ratio expresses the preference of particles for either PF or EF face and therefore may be a useful parameter for detecting alterations in the organization of a membrane.

More detailed information about the techniques, applications and interpretation of freeze-fracturing and freeze-etching may be found in various reviews and handbooks (e.g. Benedetti and Favard, 1973; Fleischer and Packer, 1974, Stolinski and Breathnach, 1975; Gulik-Krzywicki, 1975; Margaritis et al., 1977).

8.2. Methods

For the freeze-fracturing technique, small drops of concentrated membrane suspensions are mounted on golden discs and are rapidly frozen, from room temperature, by melting Freon 22, either without or after fixation with a solution of 1% glutaraldehyde and 1% paraformaldehyde in cacodylate buffer (Karnovsky, 1965). This fixation is followed by impregnation with 20% glycerol for half an hour, prior to freezing. This impregnation is omitted in unfixed preparations. No difference could be detected between electronmicrographs of fixed or unfixed preparations. The samples are stored in liquid nitrogen. Fracturing and replication is performed in a Balzer apparatus BA 360 M of BAF 300 at $-150^{\circ}C$ and an electron gun system is used for the evaporation of platinum and carbon, under a 45° angle. The replicas are examined in a Philips 300 or 301 electronmicroscope.

The procedures of sample preparatior for thin section electronmicroscopy are described in section 3.3.11.

8.3. Results and discussion

Observation of positively stained thin sections of phospholipase C treated photoreceptor membranes reveals large electron dense areas in close contact with clustered membranous structures (fig. 8.5), while untreated preparations are completely free of this material (fig. 8.4). These electron dense areas very likely represent droplets of diglycerides, resulting from the enzymatic phospholipid hydrolysis. They closely resemble the droplets resulting from phospholipase C treatment of erythrocyte ghosts (Ottolenghi and Bowman, 1970; Coleman et al., 1970; Limbrick and Knutton, 1975). Biochemical analysis of the latter droplets indicates that they are rich in neutral lipids (Coleman et al., 1970; Finean and Coleman, 1970; Allan et al., 1975). Apparently the reduction of the amphipathic character of lipids through removal of the polar head groups

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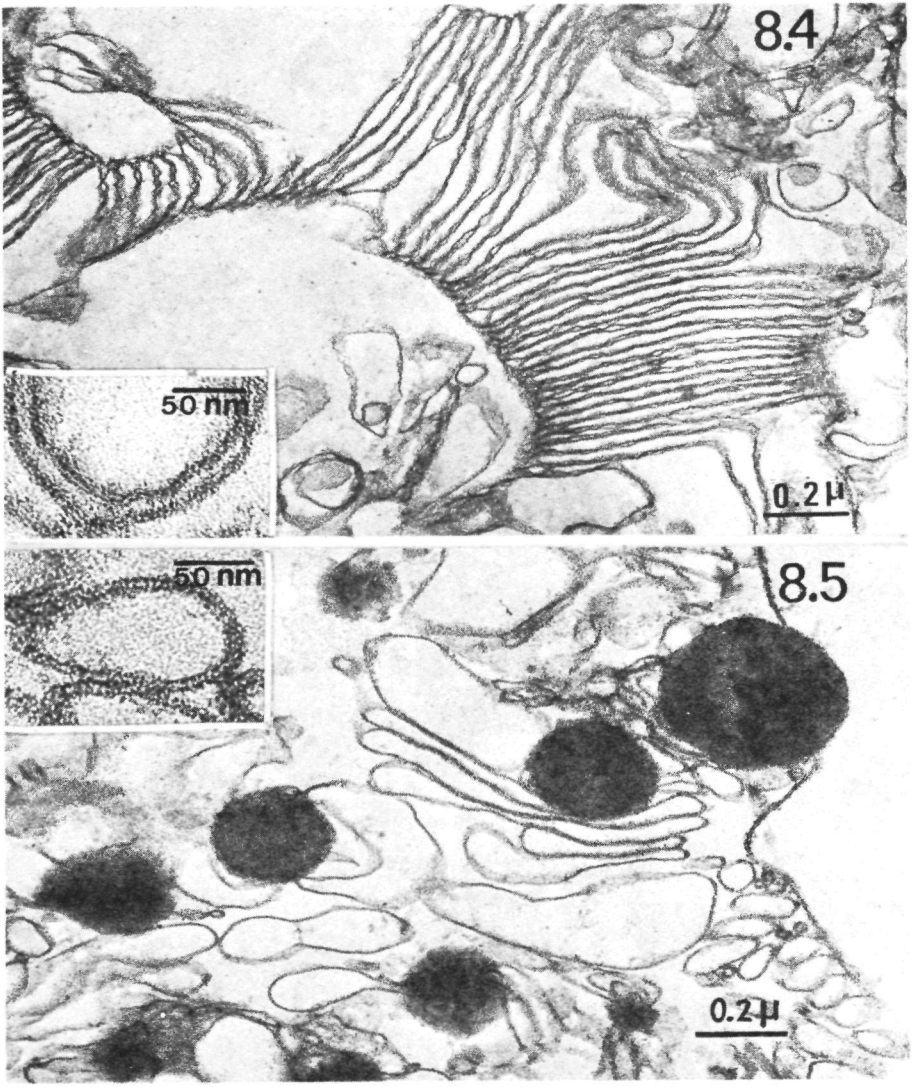
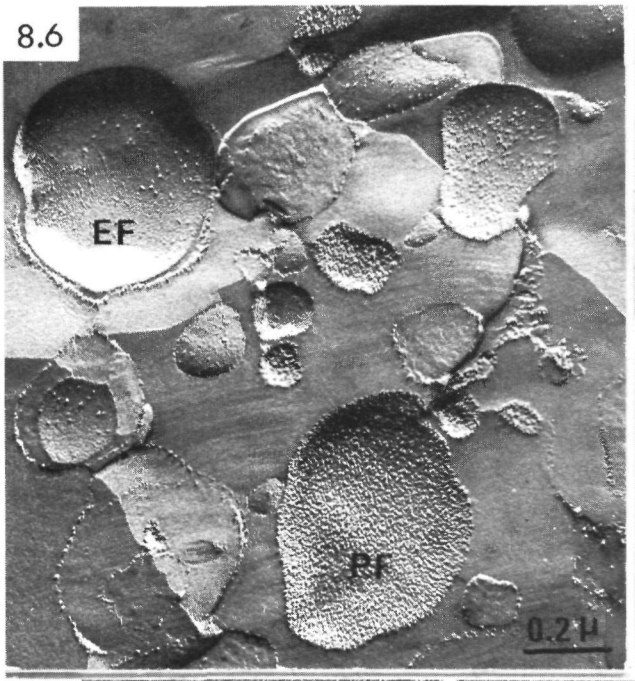
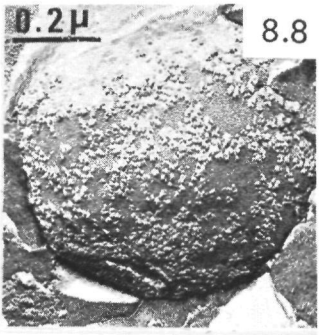


Fig. 8.4. Thin section of isolated rod outer segments exhibiting mild lysis. Insert: two membranes at higher magnification.

Fig. 8.5. Thin section of phospholipase C treated rod outer segment membranes after about 90% hydrolysis of the phospholipids. Insert: the triple layer aspect is still clearly visible.


8.6
EF
PF
0.2μ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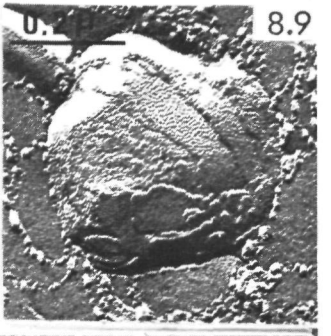
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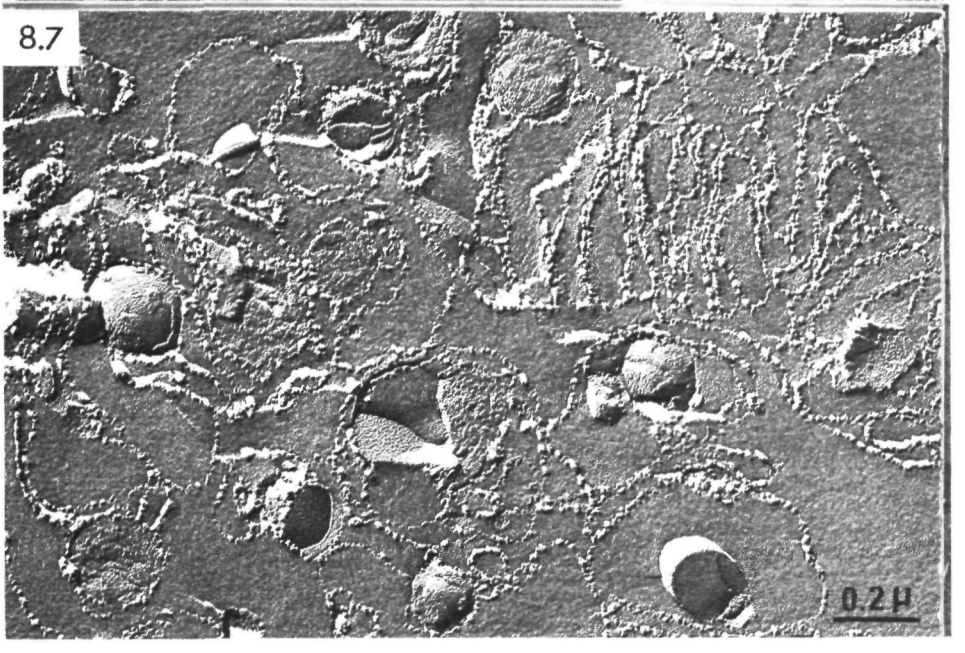
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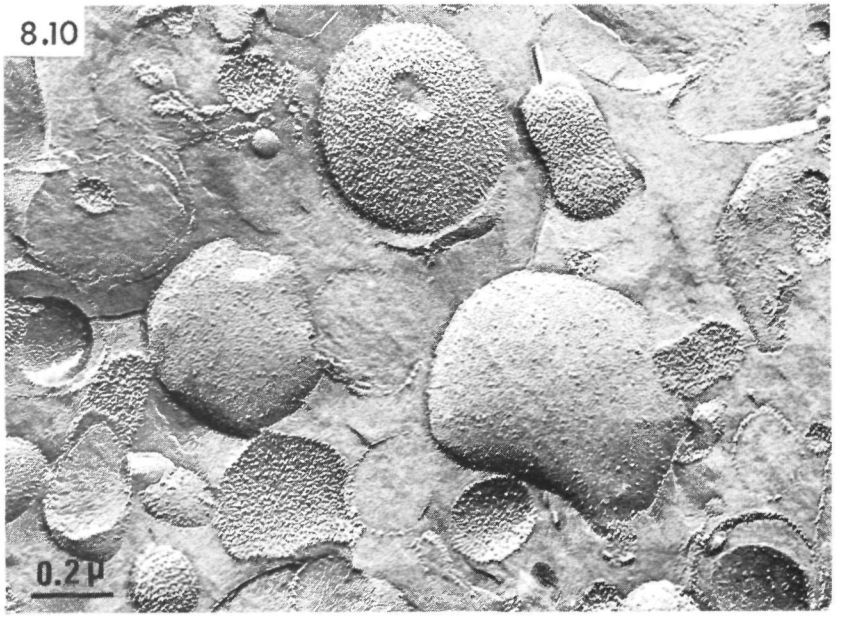

Fig. 8.6. Freeze-fractured photoreceptor membranes (untreated). For the meaning of EF and PF, as well as a description of the technique, see section 8.1. The average diameter of the particles at the PF-face is about 70 Å, their density is about 10,000 per μ^2.

Fig. 8.7. Freeze-fractured photoreceptor membranes after 90% hydrolysis of phospholipids by phospholipase C, showing lipid droplets and cross-fracturing of membranous structures.

Fig. 8.8. Freeze-fractured photoreceptor membranes after 40% hydrolysis of phospholipids, representing the rare case in which the membrane is fractured along the hydrophobic interior, with segregation of proteins and lipids.

Fig. 8.9. Freeze-fractured diglyceride droplet present in a photoreceptor membrane preparation after 90% hydrolysis of phospholipids.

Fig. 8.10. Freeze-fractured photoreceptor membranes after treatment with pronase. The SDS gel electrophoretic pattern of this preparation is shown in fig. 4.2.C. The average diameter of the particles at the particle-rich face is about 46 Å, their density is about 15,000 per μ^2.

results in their extrusion from the membrane. The diglycerides formed in one membrane, possibly together with those of proximate membranes, coalesce to spherical structures (droplets) with the least possible exposure to the aqueous phase. The process of extrusion and coalescence may start at a critical concentration of diglycerides in the membrane. In membranes, where about 20% of the phospholipids have been hydrolyzed, small droplets are occasionally seen. After 40% hydrolysis of the phospholipids distinct droplets are invariably present. Upon further hydrolysis there is a tendency towards an increase in the size of the droplets.

The size and shape of the vesicular membranous structures are too variable to allow estimation of reduction in the surface area of the membrane upon extrusion of the diglycerides. Therefore, our assumption that nearly all diglycerides are gathered in the droplets is only based on the analogy with erythrocyte membranes. Replicas of freeze-fractured phospholipase C treated photoreceptor membranes show droplets of similar size and frequency as found in the thin sections (figs. 8.5 and 8.7). There is some indication of a lamellar structural organization within these droplets (fig. 8.9), which is comparable to that of neutral lipid droplets in foam cells (Hasegawa and Uyeda, 1974), liver cells (Stolinski and Breathnach, 1975) and in milk (Buchheim, 1970). No inclusions are seen with the dimensions of the intramembranous particles (probably monomers or oligomers of rhodopsin) visible on fracture-faces of untreated preparations (PF in fig. 8.6) and on the edges of cross-fractured phospholipase C treated membranes (figs. 8.7 and 8.9).

The enzymatic hydrolysis of the phodpholipids into water-soluble phospnate esters and diglycerides thus appears to result in the effective removal of phospholipids from the membranous structure. Consequently, all rhodopsin molecules must reside in the residual membranous structures that are observed in thin sections of membranes treated exhaustively with phospholipase C (about 90% phospholipid hydrolysis). These structures seem to be more dilated and fragmented than those in untreated preparations. The triple layered aspect of membranous structures after positive staining is even more pro-

nounced in the lipid depleted membranes than in the native membranes (inserts in figs. 8.4 and 8.5). This is a remarkable observation, and the question arises whether these membranous structures do still contain a lipid bilayer. After 90% phospholipid hydrolysis only 7 phospholipid molecules remain per rhodopsin molecule. Calculation shows that a hexagonal packing of phospholipids (80 $\AA^2$ cross-sectional area, Demel et al., 1972) in bilayer arrangement leaves space for a protein molecule of 10 Å diameter. Since the diameter of a rhodopsin molecule is probably much larger (approximately 50 Å, Daemen, 1973; Corless et al., 1976), complete surrounding of all rhodopsin molecules by lipids appears unlikely. The loss of hydrophobic shielding by the lipids may thus be compensated by mutual lateral contacts between the hydrophobic surfaces of rhodopsin molecules, rather than by a breaking-up of their two-dimensional arrangement. It should be remembered that rhodopsin is the major membrane protein, representing at least 85% of all protein present in the photoreceptor membrane (cf. fig. 4.2). We may thus conclude that the membranous structures observed after exhaustive treatment of photoreceptor membranes with phospholipase C represents mainly lateral aggregates of rhodopsin molecules.

This view seems to be supported by the observation that freeze-fracture of preparations subjected to extensive phospholipid hydrolysis invariably leads to cross-fracturing of the membranous structures and to appearance of particles at the edges of the cross-fractured structures (figs. 8.7 and 8.9). This is in contrast to untreated membranes, which are usually fractured along their internal hydrophobic core (fig. 8.6). These observations indicate that after phospholipase C treatment a lipid bilayer does no longer determine the nature of membrane cleavage. This is also observed in phospholipase C treated erythrocyte membranes (Limbrick and Knutton, 1975).

Even at 40% phospholipid hydrolysis cross-fracturing is frequently observed. In the rare cases where fracturing along the hydrophobic interior of the membrane occurs, random lateral dispersion is no longer maintained and segregation between lipids (smooth areas) and particles is observed (fig. 8.8). Segregation

is a well known phenomenon and may result from temperature sensitive lateral phase separations (see section 2.2.6), as shown for rhodopsin in artificial membranes (Chen and Hubbell, 1973). It may also be initiated by proteolytic or lipolytic treatment of biological membranes (e.g. Speth et al., 1972; Limbrick and Knutton, 1975). However, in our experiments pronase treatment of photoreceptor membranes does not give rise to segregation (cf. fig. 8.10). The exposure of hydrophobic surfaces upon freeze-fracturing of membranes after intermediate levels of phospholipid hydrolysis is too rarely observed to justify the conclusion that in the unrevealed membranes segregation occurs also.

In the final chapter the effects of alteration of the microenvironment of rhodopsin on photolytic behaviour (chapter 6), thermal stability and regeneration capacity (chapter 7) will be interpreted in the light of the present electronmicroscopic observations, which strongly suggest that exhaustive treatment of photoreceptor membranes with phospholipase C leads to the formation of two-dimensional (lateral) aggregates of rhodopsin molecules.

CHAPTER 9

GENERAL DISCUSSION. INTERACTIONS BETWEEN LIPIDS AND RHODOPSIN

9.1. Normal functioning of rhodopsin requires a lipid bilayer

The photolytic behaviour (chapter 5 and 6) and the thermal stability as well as the regeneration capacity (chapter 7) of various rhodopsin preparations show parallel responses to experimental alteration of the micro-environment of the visual pigment molecule, i.e. alteration of the lipid complement of the photoreceptor membranes.

Phospholipids may be removed from rhodopsin in two independent ways. One procedure consists of solubilization of the photoreceptor membranes in a solution of the cationic detergent dodecyltrimethylammonium bromide (DTAB, fig. 9.1, A → B), followed by affinity chromatography over concanavalin A-sepharose 4B which removes all lipids and probably also all proteins other than rhodopsin (fig. 9.1, B → C, see section 3.3.7). Subsequent dialysis of the detergent results in a suspension of lipid- and detergent-free rhodopsin (fig. 9.1, C → D). Phospholipase C treatment of photoreceptor membranes is an effective way for partial removal of phospholipids from the membranes, as concluded in section 8.3 from the observed extrusion of diglycerides from residual membranous structures (fig. 9.1, A → D). These two procedures of delipidation affect rhodopsin in identical fashion. In lipid- and detergent-free rhodopsin as well as in photoreceptor membranes exhaustively treated with phospholipase C (ca. 90% phospholipid hydrolysis) the photolytic sequence does no longer reach the normal terminal stage of metarhodopsin III and free retinaldehyde, but appears to be largely blocked at the

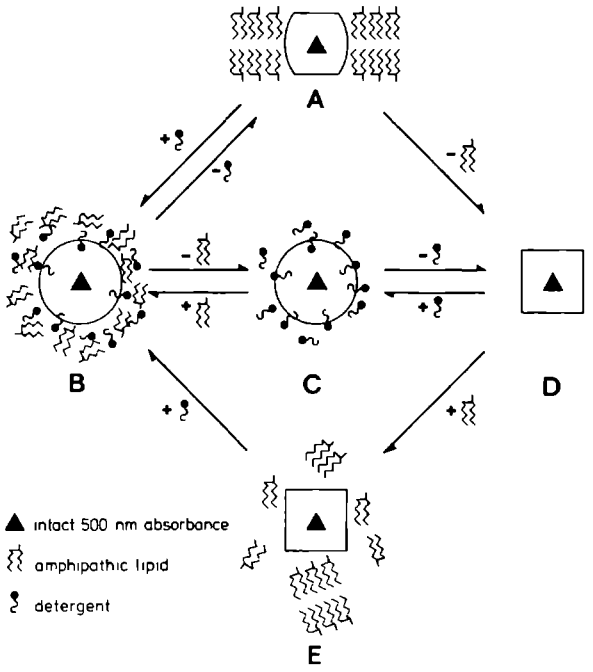

Fig. 9.1. Schematic presentation of the interrelations between various rhodopsin preparations. A. Functionally intact rhodopsin embedded in a lipid bilayer of either the native membranes or those obtained after reconstitution with amphipathic lipids (B → A). B. Rhodopsin in detergent solution in the presence of phospholipids. The circular shape of rhodopsin stands for the mobilized, detergent penetrated protein structure. C. Detergent solubilized rhodopsin obtained after affinity chromatography (B → C). D. The square protein symbol represents the more rigid structure in (lateral) aggregates of rhodopsin obtained either after detergent dialysis of lipid-free rhodopsin solutions (C → D) or by reducing the amphipathic character of the phospholipids by phospholipase C treatment of native membranes (A → D). E. Phospholipids mixed with preparation D without detergent.

metarhodopsin I to II transition (chapter 6). Similar observations have been reported for lipid- and detergent-free rhodopsin by Applebury et al. (1974). The regeneration capacity in both preparations is about 50% whereas a value of about 90% is found in native membrane preparations (chapter 7). Hong and Hubbell (1973) have re-

ported a comparable decrease in regeneration capacity of lipid- and detergent-free rhodopsin suspensions The thermal stability of rhodopsin in both delipidated preparations is also clearly decreased, although somewhat less for the phospholipase C treated preparation than for lipid- and detergent-free rhodopsin (chapter 7).

These effects, which are obviously due to phospholipid removal, can be completely reversed by reconstitution of the rhodopsin preparation with amphipathic lipids. However, this requires the intermediate use of detergent (fig. 9.1, D → C → B → A). All amphipathic lipids tested (see table 6.4) are equally suitable for the restoration of the normal photolytic behaviour of rhodopsin (see section 6.4.3). The same is found for the regeneration capacity, except that here didecanoylphosphatidylcholine is somewhat less effective than other amphipathic lipids (see section 7.3.2; cf. Hong and Hubbell, 1973). The restoration of the thermal stability has only been tested for egg phosphatidylcholine, employing the detergent dialysis procedure for reconstitution with rhodopsin. The fact that this reconstitution is successful supports the findings made for the other two parameters.

Mere addition of amphipathic lipids to affected rhodopsin preparations without the intermediate use of detergent (fig. 9.1, D → E) in general does not result in restoration of the normal photolytic behaviour of rhodopsin (see also Applebury et al., 1974). Likewise, this does in general not restore the regeneration capacity. Reconstitution of preparations of delipidated rhodopsin with neutral lipids (oleoyl - glycerides), either by the detergent dialysis procedure or by mere addition, never reverses the changes in properties of these rhodopsin preparations.

The results of spin label measurements (Hong and Hubbell, 1972) as well as of freeze-fracture electronmicroscopic studies (Chen and Hubbell, 1973) strongly suggest that rhodopsin becomes reincorporated into a lipid bilayer, when it is reconstituted with amphipathic lipids by means of the detergent dialysis procedure. We have confirmed the appearance of intramembranous particles upon freeze-fracturing of dioleoylphosphatidylcholine-rhodopsin reconstituted

membranes (unpublished results). Application of the same technique of lipid-protein reconstitution (intermediate use of DTAB) has also been shown to result in reincorporation of some erythrocyte membrane proteins into artificial lipid bilayers (Grant and Mc Connell, 1974, Barratt et al., 1977). Glycerides do not provide a lipid bilayer. Therefore, we can conclude that rhodopsin must be incorporated in a lipid bilayer in order to exhibit normal photolytic behaviour, regeneration capacity and thermal stability. However, the nature of the polar head groups of the amphipathic lipids does not seem to be of importance.

The analogy in behaviour between lipid- and detergent-free rhodopsin and rhodopsin in photoreceptor membranes exhaustively treated with phospholipase C strongly suggests that detergent induced artifacts are absent in lipid- and detergent-free rhodopsin. Such effects cannot have occurred in phospholipase C treated membranes. The parallellism between the abnormalities of rhodopsin makes it also plausible that they have a similar origin in both preparations.

The concept of aggregation of rhodopsin molecules can most easily explain why phospholipid removal has deleterious effects, why detergent is needed to reverse the situation and furthermore, why the regeneration capacity is never completely lost. Electronmicroscopic studies seem to justify the conclusion that lateral aggregates of rhodopsin molecules are formed during phospholipase C treatment (see section 8.3). Lipid- and detergent-free rhodopsin appears as amorphous material in thin section electronmicroscopy (unpublished results). Undoubtedly aggregation also occurs, although it may not be restricted to lateral contacts.

It is generally believed that the metarhodopsin I to II transition is accompanied by relatively large conformational changes within the protein, while simultaneously a proton is taken up (see section 1.4.3). When the loss of hydrophobic shielding by phospholipids is compensated by (lateral) interactions between the (hydrophobic) surfaces of rhodopsin molecules, the metarhodopsin I decay is strongly hampered. Aggregated rhodopsin molecules may be immobi-

lized and may also have lost freedom of internal motion or even parts of the rhodopsin molecule may be structurally reorganized. Either one of these changes or a combination of them obviously hampers the conformational changes normally accompanying the metarhodopsin I to II transition. If structural reorganization of the rhodopsin molecule does occur, it must be restricted to those parts of rhodopsin that are not involved in the construction of the chromophoric centre, since the typical rhodopsin absorbance spectrum is not at all affected by delipidation. Insufficient hydrophobic shielding and resulting exposure of parts of the hydrophobic surface of rhodopsin to the aqueous phase would demand compensatory changes in the protein, in order to overcome the otherwise abnormal polarity difference between the unmasked surface and the surrounding fluid. The observation that removal of amphipathic lipids results in increased susceptibility of rhodopsin to proteolytic enzymes (chapter 4) may be an indication for inadequate shielding. Direct proof for structural reorganization has, however, not been obtained.

Aggregation without conformational changes may equally well account for the dysfunctioning of the photolytic sequence. The protein may become more rigid and thereby lose the flexibility to undergo the normal conformational changes accompanying the metarhodopsin I to II transition. This possibility is supported by the observation that treatment of rod outer segment membranes with the bifunctional (cross-linking) agent glutaraldehyde causes the same change in the photolytic behaviour of rhodopsin as is found in the delipidated preparations (unpublished results). This does not seem to be due to the modification of primary amino groups, since their complete modification does not affect the formation of the metarhodopsin II (De Grip, 1974; see section 6.4.2). Glutaraldehyde treatment results in an inhibition of the rotational movement of rhodopsin (cf. Brown, 1972), but it may also increase the internal rigidity of the protein (by intramolecular cross-linking). Dry lyophilized rod outer segment membranes also display inhibition of metarhodopsin I decay (Wald et al., 1950). Therefore, absence of water at appropriate places or inaccessibility of rhodopsin for

water (and protons), needed for the metarhodopsin I decay, may also play a role in the abnormal photolytic behaviour of aggregated rhodopsin molecules.

The thermal stability is decreased after removal of amphipathic lipids (see chapter 7). This indicated an unfavourable condition of the rhodopsin molecules, which is apparently somewhat worse for purified rhodopsin than for rhodopsin in membranes treated exhaustively with phospholipase C. In the latter preparation rhodopsin may have some structural support from the residual lipids, but the observations do not indicate a specific molecular mechanism.

The effects of amphipathic lipid removal on the regeneration capacity favour the interpretation that mere aggregation of the rhodopsin molecules without their structural reorganization is the underlying mechanism, if we assume that part of the opsin molecules are inaccessible to 11-cis retinaldehyde, because the chromophoric binding side is shielded by adjacent opsin molecules. Regeneration of rhodopsin is an all-or-none process, i.e. a molecule is either regenerated or it is not. The above interpretation thus avoids the only alternative explanation that as a result of delipidation 50% of the opsin (rhodopsin) molecules is more seriously damaged than the other 50%. If shielding does indeed account for the decreased regeneration capacity, it follows that not all opsin molecules are shielded. Furthermore, this situation must be rather static, since incubation of the illuminated aggregated rhodopsin preparations with 11-cis retinaldehyde for longer than 1.5 hr does not cause additional regeneration. Therefore, the lateral and rotational movements of the protein molecules within the aggregates are restricted in such a way that an inaccessible opsin molecule remains in this condition. This interpretation leads to the important conclusion that the removal of amphipathic lipids has no impact on the conformation of each individual protein molecule, as judged by the regeneration capacity.

To achieve proper reconstitution of rhodopsin in a lipid bilayer, the intermediate application of detergent is required (see also Applebury et al., 1974). This suggests that detergents can

easily break up aggregates, while amphipathic lipids generally cannot. Addition of detergent to phospholipase C treated membranes or to suspensions of lipid- and detergent-free rhodopsin, which are in the (blocked) metarhodopsin I state, abolishes this blockade. In addition to causing disaggregation, the detergent could also increase the flexibility of the protein molecule, thereby offering the structurally affected rhodopsin molecules a chance to regain their proper conformation. Only when the detergent is removed in the presence of amphipathic lipids, this conformation is retained. This suggests that specific areas of rhodopsin require hydrophobic interactions, which do not impose rigidity, while other parts of the molecule require polar interactions. These conditions are apparently only provided by a lipid bilayer, in which a rhodopsin molecule can be oriented according to the polarity distribution of its surface.

The occasional restoration of normal photolytic behaviour and regeneration capacity upon mere addition of photoreceptor membrane lipids to membranes treated exhaustively with phospholipase C, may be due to the existing two-dimensional structure, where the residual phospholipids may guide the added phospholipids in between the aggregated rhodopsin molecules. These observations strengthen our hypothesis that the changes in the properties of rhodopsin are caused by mere aggregation without structural reorganization of the protein (rigid state), and can be reversed by disaggregation (flexible state).

A similar explanation may be given for the effects of the treatment of erythrocyte membranes by phospholipase C (Roelofsen and Schatz mann, 1977). In that case there is a parallel decrease in the Ca^{2+} ATPase activity and the percentage degradation of the glycerophospholipid fraction in the inner membrane layer. The maximal extent of removal of amphipathic lipids is, however, much less than that found in rod outer segment membranes treated extensively with phospholipase C. This may explain why it is found that mere addition of glycerophospholipids can already restore the Ca^{2+} ATPase activity, independent of th nature of the polar head group of these lipids.

Didecanoylphosphatidylcholine is the most detergent-like lipid of the series used in our studies. The occasional restoration of the original properties of rhodopsin upon mere addition of this lipid indicates that it is the most potent in breaking up rhodopsin aggregates.

9.2. Requirement of a lipid annulus

Phospholipase C treatment can lower the phospholipid/rhodopsin molar ratio in the rod outer segment membrane from 70 to 7. It would be possible in principle to estimate the minimal ratio needed for proper functioning of rhodopsin. The photolytic behaviour (fig. 6.2.A) and regeneration capacity (fig. 7.1) are detectably affected, only when more than about 40% of the phospholipids are hydrolyzed, and therefore a minimal molar phospholipid/rhodopsin ratio of about 40 seems to be required. However, this value may not be meaningful. Phospholipase C treatment can induce segregation between protein particles and lipids even at 40% phospholipid hydrolysis (see fig. 8.8). We have not been able to conclude that segregation always occurs at such intermediate levels of phospholipid hydrolysis (see section 8.3). However, if segregation occurs, the actual molar phospholipid/rhodopsin ratio within the patches of clustered particles will be much lower than that of the preparation as a whole. The minimal ratio of 40, therefore, is only valid if the rhodopsin molecules are randomly distributed.

The properties of rhodopsin are not yet affected at 40% phospholipid hydrolysis. As discussed in section 9.1, we attribute the effect of phospholipase C treatment to the formation of rhodopsin aggregates. Thus, if at 40% hydrolysis all rhodopsin molecules are involved in segregation, it follows that segregation and aggregation are two different phenomena. It then also follows that the number of phospholipid molecules, required for maintaining the normal properties of the visual pigment, will be far below 40.

Both regeneration and blocking of the metarhodopsin I to II transition are most likely all-or-none processes. Therefore, the observation that a gradual increase of phospholipid hydrolysis is accompanied by a gradual decrease in the number of normally functioning molecules can be explained by a gradual increase of aggrega-

tion either of randomly distributed or of segregated pigment molecules.

Obviously rhodopsin does need a lipid annulus for normal functioning. The required number of phospholipid molecules per rhodopsin molecule is probably less than 40. A more precise estimation of the minimal number may be obtained by studying the properties of rhodopsin in reconstituted membranes in dependence on molar phospholipid/rhodopsin ratios between 7 and 40.

9.3. Effect of membrane fluidity

The phospholipids of the photoreceptor membrane are highly unsaturated (see section 1.3.2). None of the phospholipids used in our reconstitution experiments is completely saturated, except for didecanoylphosphatidylcholine. Their unsaturation is, however, definitely lower than that of rod outer segment membrane lipids. Nevertheless, we find that all amphipathic lipids provide an equally suitable environment of rhodopsin. This does not mean that the properties of rhodopsin are independent of the ordening of the lipid chains, since at 25^{o}C most of the amphipathic lipids used in our studies are probably above their transition temperature. Nevertheless, we may conclude that none of the properties of rhodopsin that we have studied, requires the uniquely high degree of unsaturation found in native photoreceptor membranes.

If our hypothesis is right that increased rigidity of the protein molecule (in the aggregated state) is responsible for blocking the metarhodopsin I decay, while the regeneration is only affected due to shielding and not to conformational changes, then it will be interesting to look at the properties of rhodopsin in lipid bilayers composed of phospholipids with rather high transition temperatures. Below the transition temperature the rigidity of the fatty acid chains might impose rigidity on the rhodopsin molecules and this can be detected by the photolytic behaviour. If the protein molecules remain randomly distributed under such conditions, which can be

detected by freeze-fracturing, then the regeneration capacity should not be affected.

9.4. Dependence on membrane thickness

The regeneration capacity of rhodopsin-didecanoylphosphatidylcholine reconstituted membranes is lower than that observed in membranes formed by phospholipids with greater fatty acid chain-length. This has been explained by the assumption that the didecanoylphosphatidylcholine bilayer is too thin to accomodate the protein molecule (see Hong et al., 1973, Chen and Hubbell, 1973). This interpretation is not supported by our observations. Didecanoylphosphatidylcholine restores the photolytic behaviour to the same degree as all other amphipathic lipids studied (chapter 6). This parameter reflects to some extent the constitution of the rhodopsin molecule, while the regeneration capacity does not. Therefore, the slightly lower regeneration capacity of the rhodopsin-didecanoylphosphatidylcholine reconstituted membranes must have another cause than inadequate hydrophobic shielding of the rhodopsin molecules.

9.5. Phospholipid asymmetry

In section 3.3.10 we reported that the results of phospholipase C treatment of rod outer segment membranes do not give a clear indication for or against phospholipid asymmetry. Nevertheless, phospholipid asymmetry may have important implications, since one of the basic assumptions of the current hypothesis of visual excitation implies steep calcium gradients over the photoreceptor membranes, at least in the dark-adapted state (see fig. 1.9). It is very well conceivable that an asymmetric distribution of, notably, phosphatidylserine, might function in the storage of calcium inside the rod discs. In addition, the role of phosphatidylethanolamine in the formation of metarhodopsin III (chapter 6) requires further study. In

native photoreceptor membranes all rhodopsin molecules are identically oriented (see section 2.2.4), while in artificial membranes, obtained by reconstitution (detergent dialysis procedure) of rhodopsin and photoreceptor membrane lipids, the rhodopsin molecules (and phospholipid molecules) can be expected to be randomly oriented (cf. Chen and Hubbell, 1973). The observation that in both types of membranes the fraction of retinylidene-phosphatidylethanolimine in the final photoproducts is equal (section 6.3.1), is then most easily explained by a completely asymmetric distribution of phosphatidylethanolamine in native photoreceptor membranes.

The negative results of phospholipase C treatment in demonstrating phospholipid asymmetry in photoreceptor membranes may be explained by the drastic desintegration of the lipid bilayer of these membranes upon this lipolytic treatment (chapter 8). Therefore, treatment with lipolytic enzymes which better preserve the amphipathic character of the membrane phospholipids might be useful. Phospholipase C seems a suitable candidate, since it can convert all glycerophospholipids into the amphipathic phosphatidic acid by splitting off the terminal alcoholic group (X in fig. 1.4). In addition, we have shown that reconstitution with pure phosphatidic acid provides a suitable membrane environment for rhodopsin, as judged by photolytic behaviour and regeneration capacity (see table 6.3 and section 7.4). A reliable method to follow the enzymatic hydrolysis quantitatively is available two-dimensional thin-layer chromatography.

However, four conditions have to be fulfilled in order to arrive at valid conclusions. First, phosphatidic acid should not exhibit so-called "flip-flop"-movement from one side of the membrane to the opposite one. Secondly, the membrane vesicles exposed to phospholipase D should remain impermeable for this enzyme. These two conditions can be checked by estimating the maximal degree of phospholipid hydrolysis, which should not exceed 50%. Thirdly, effects of a possible preference of phospholipase D for specific types of phospholipids should be eliminated. This may be achieved by comparing the enzymatic hydrolysis of native membranes to that

of artificial membranes obtained from lipids of photoreceptor membranes and rhodopsin, since in these reconstituted membranes probably no asymmetry exists (see above). Finally, it should be ascertained that the native membrane vesicles used as a substrate have identical outside faces with regard to the orientation of the rhodopsin molecules. Here, the sugar residues of rhodopsin may be used to recognize one particular side of the photoreceptor membrane (probably the intradiscal side) by application of (immobilized) concanavalin A.

Other enzymes, which may turn out to be useful in resolving a possible phospholipid asymmetry of photoreceptor membranes, are phospholipase A_2 (Zwaal et al., 1975) and phosphatidylserine-decarboxylase. The former removes a fatty acid from the 2-position of glycerophospholipids, whereas the latter specifically converts phosphatidylserine into phosphatidylethanolamine.

9.6. Concluding remarks

One of the most intriguing conclusions of this thesis is that none of the presently known properties of rhodopsin requires a specific composition of the lipid bilayer surrounding the pigment molecule. However, it is difficult to accept that especially the uniquely high degree of phospholipid unsaturation, would not have functional significance (see section 1.3.2). Presumably, the typical composition of the photoreceptor membrane lipids is important for other, as yet unassessed functions of rhodopsin or the membrane. Considering that an obvious deficiency in our knowledge of the visual process concerns the coupling between rhodopsin photolysis and the translocation of ions during the transduction process, it is tempting to suggest that the presently unknown output signal of rhodopsin photolysis requires a specific phospholipid unsaturation. Therefore, it seems important to study the permaphore character of the visual pigment in photoreceptor membranes and in membranes reconstituted with specific amphipathic lipids.

The question whether or not rhodopsin spans the entire width of the membrane, is related to its possible role as permaphore. The present studies of proteolytic treatment of photoreceptor membranes are not conclusive in this respect, since the sidedness of the membrane exposed to the proteases is not definitely settled. Prior separation between membrane vesicles which are everted and those which are not may be essential and is feasible by affinity chromatography. Subsequent exposure to proteolytic enzymes and analysis of the fragmentation pattern should reveal differences, which could lead to definite conclusions about the exposure of parts of rhodopsin at both sides of the membrane.

An additional approach to the question whether rhodopsin spans the membrane is offered by the freeze-etching technique, using both native and reconstituted membranes before and after protease treatment. Since the sugar residues of rhodopsin remain attached to the major proteolytic fragments, the side of location of the carbohydrate moieties may be revealed after freeze-etching, if they are properly labeled, e.g. by conjugates of concanavalin A and ferritin or hemocyanin.

Finally, it should be mentioned that studies of the photoreceptor membrane are not only important for a better insight in the molecular mechanism of the visual process, but also for a deeper understanding of the interrelations between proteins and lipids in biological membranes in general. The photoreceptor membrane presents an excellent experimental object for such studies in view of the natural probe character of rhodopsin, the relative ease of large scale isolation of photoreceptor membranes and pure rhodopsin and also the possibility to incorporate rhodopsin into artificial membranes.

In addition to general cellular functions, the photoreceptor cell contains the complete system for photon absorption and for transduction and amplification of the signal, eventually leading to its synaptic transmission. The photon absorption system is entirely located in the outer segment of the photoreceptor cell. In rod cells, to which the present study has been confined, it is organized in a pile of stacked discs. The discs consist of flat, closed "photoreceptor membranes". Signal transduction and amplification must be the result of an interplay between the photoreceptor membrane and the surrounding plasma membrane, probably through light-induced release of calcium ions from the discs leading to decrease in sodium permeability of the plasma membrane. The key molecule in the visual process is the visual pigment rhodopsin. Its absorption of an incident photon starts off the whole sequence of events between light absorption and light perception in the central nervous system.

Rhodopsin is an intrinsic membrane protein present in high concentration in the photoreceptor membrane. It has a characteristic absorbance spectrum (λ_{max} = 500 nm) and contains the 11-cis isomer of retinaldehyde as chromophore. This isomer is, upon illumination of rhodopsin, converted to its all-trans form. This light-induced conversion leads to a sequence of spectral transitions, which together are often called the photolysis of rhodopsin.

The present study focuses on the organization of the rod photoreceptor membrane. The unique composition of this membrane must have a functional background and, therefore, we have emphasized the study of interactions between the major components of this membrane· the membrane protein rhodopsin and the phospholipids, which provide the bilayer backbone of the membrane.

In chapter 1 our present understanding of the structure and function of the rod photoreceptor cell is reviewed, with special reference to structure and composition of the outer segment, to the properties of rhodopsin as a visual pigment and to current views of the visual excitation mechanism.

In chapter 2 present ideas on structural and dynamic properties of the rod photoreceptor membrane are discussed in more detail. Here, the properties of rhodopsin as an intrinsic membrane protein are presented and the aim of our study is defined.

In chapter 3 a description is given of materials, procedures and determinations, which have been used throughout this study, including the elaboration of a method to purify rhodopsin by means of affinity chromatography.

In chapter 4 we report our contributions to the topography of rhodopsin and other proteins in the photoreceptor membrane, in which rhodopsin comprises over 85% of total membrane protein. Photoreceptor membrane suspensions are subjected to treatment with proteases (trypsin, α-chymotrypsin, papain, subtilisin and pronase). All essential properties of rhodopsin appear to be resistant against this treatment. Nearly complete preservation is found of its characteristic absorbance spectrum, as well as its photolytic behaviour, thermal stability and regeneration capacity (degree of reconversion of illuminated pigment into rhodopsin by treatment with 11-cis retinaldehyde). Nevertheless, gel electrophoretic analysis shows that a substantial part (> 25%) of rhodopsin can be digested away by all enzymes, except by trypsin. When the structural organization of the photoreceptor membrane is disturbed by addition of detergent, or by phospholipase C treatment or when rhodopsin is completely delipidated, the essential properties of rhodopsin are no longer resistant against the proteases. These observations indicate that all protein parts involved in the maintainance of the known characteristics of rhodopsin are shielded by lipids of the photoreceptor membrane and that rhodopsin is thus largely embedded within the hydrophobic core of the lipid bilayer.

In order to investigate whether the functional and structural properties of rhodopsin depend on the presence and composition of a lipid bilayer, it is essential to change the micro-environment of the pigment molecule. This can be achieved in different ways. First, by acetylation of primary amino groups of the phospholipids of the photoreceptor membrane. Secondly, by treatment of these membranes with phospholipase C, a lipolytic enzyme which hydrolyses phospholipids to diglycerides and water-soluble phosphate esters, and thus reduces the amphipathic character of the lipids. Evidently, complete delipidation of rhodopsin by means of affinity chromatography implies a drastic alteration of its micro-environment. Finally, we can replace all original lipids by selected lipids by means of a detergent dialysis procedure. When amphipathic lipids are used, this procedure results in the reincorporation of rhodopsin in a lipid bilayer. The effects of these changes in the micro-environment of rhodopsin on various properties of the visual pigment (photolytic behaviour, thermal stability and regeneration capacity) can then be studied.

In chapter 5 the late and slower part of the photolytic sequence of rhodopsin in the photoreceptor membrane is studied. Rapid conversion of metarhodopsin I to metarhodopsin II is observed, while metarhodopsin II undergoes a slow conversion in two directions: hydrolysis to all-trans retinaldehyde and opsin and transiminization of retinaldehyde to other amino groups on either opsin or phosphatidylethanolamine. The transiminized intermediates are found to represent "metarhodopsin III".

In chapter 6 we use the results of the preceding chapter in order to investigate the effect of changing the micro-environment on the photolytic behaviour of rhodopsin. Phospholipase C treatment of photoreceptor membranes and complete delipidation of rhodopsin cause blocking of the metarhodopsin I to II transition. The normal photolytic behaviour is recovered, when the pigment molecule is reincorporated into a lipid bilayer. The nature of the polar head groups of the amphipathic lipids appears to be of no importance.

In chapter 7 we describe the effects of modification of the micro-environment of rhodopsin on thermal stability and regeneration capacity. The changes in these two parameters also reveal deleterious effects of phospholipase C treatment or complete delipidation. These effects are reversed by reincorporation of rhodopsin in a lipid bilayer. The nature of the polar head groups of the amphipathic lipids is again not critical.

In chapter 8 we report results of electronmicroscopic studies of phospholipase C treated membranes. It is concluded that phospholipids are effectively removed from the membrane and that two-dimensional (lateral) aggregates of rhodopsin molecules are formed as a result of the lipolytic treatment.

Finally, in chapter 9 we present an integrated discussion of the results of the previous chapters. We argue that the formation of rhodopsin aggregates is most likely responsible for the changes caused by phospholipase C treatment and delipidation. We tentatively conclude that phospholipid removal causes rigidity rather than structural deformation in the rhodopsin molecules. The only requirement for the micro-environment seems to be that it preserves the internal flexibility of the pigment molecule. This condition is apparently fulfilled whenever rhodopsin is embedded in a lipid bilayer, regardless of the nature of the amphipathic lipids.

The above conclusion implies that none of the presently known properties of rhodopsin requires the uniquely high degree of phospholipid unsaturation, present in natural photoreceptor membranes. Presumably, the typical composition of the photoreceptor membrane lipids, with regard to polar head groups and unsaturation, must be important for other, as yet unassessed functions of rhodopsin or the membrane. Extension of these studies would thus seem to be important, not only for a better insight in the molecular mechanism of the visual process but also for a deeper understanding of the interrelations between protein and lipids in biological membranes in general. The photoreceptor membrane presents an excellent experimental object for such studies in view of the natural probe character of rhodopsin, the relative ease of large scale isolation of photoreceptor membranes

and pure rhodopsin and also the possibility to incorporate rhodopsin into artificial membranes.

De lichtgevoelige cellen (fotoreceptor cellen) van de retina bevatten, naast algemeen voorkomende systemen voor het celmetabolisme, een gespecialiseerd systeem dat licht in een andere vorm van energie kan omzetten, gepaard gaande met een versterking van het signaal en het doorgeven ervan naar het synaptisch gedeelte van de cel. Het buitensegment van fotoreceptorcellen bevat het gehele licht-absorberende systeem en bestaat, in staafjescellen, uit een groot aantal boven elkaar gelegen schijfjes. Deze schijfjes zijn in feite vlakke gesloten membranen en de wisselwerking tussen deze membranen en het omgevende buitenmembraan verzorgt de overdracht en versterking van prikkels, die waarschijnlijk het gevolg zijn van door licht-absorptie optredende veranderingen in de concentratiegradienten van ionen over deze membranen.

Het meest essentiele onderdeel van het visuele systeem in de staafjescel is het pigment rhodopsine. Immers, aangezien alleen dit eiwitmolekuul in staat is om zichtbaar licht te absorberen, staat het aan het begin van het hele proces dat volgt op belichting van een staafjescel en dat uiteindelijk leidt tot perceptie van "licht" in de hersenen.

Rhodopsine is een membraan-intrinsiek eiwit, d.w.z. het is niet anders dan door middelen, die het membraan verstoren, ervan los te maken. Het heeft een karakteristiek absorptie spektrum (λ_{max} = 500 nm) en bevat 11-cis retinaldehyde als chromofoor. Onder invloed van licht gaat dit isomeer over in de all-trans vorm. De lichtreaktie gaat vergezeld van opeenvolgende veranderingen in het absorptie-spektrum van het pigment.

De studie die in dit proefschrift is vervat, heeft zich gekoncentreerd op de struktuur van het fotoreceptor membraan van

staafjescellen. De aandacht is daarbij voornamelijk gericht op de wisselwerking tussen de voornaamste onderdelen van het fotoreceptor membraan. rhodopsine en fosfolipiden. Deze laatste zorgen voor de basisstruktuur van een membraan, nl. de lipide dubbellaag.

In hoofdstuk 1 wordt getracht een overzicht te geven van de huidige kennis omtrent de struktuur en het funktioneren van de staafjescel. Speciale aandacht is gewijd aan de bouw en samenstelling van het buitensegment. Tevens worden de eigenschappen van rhodopsine besproken voor zover die samenhangen met de lichtgevoeligheid van dit eiwit. Ook wordt de momenteel gangbare hypothese over het molekulaire mechanisme van de visuele excitatie behandeld.

In hoofdstuk 2 komen de huidige inzichten in de struktuur en de dynamiek van het fotoreceptor membraan meer gedetailleerd aan de orde. Die eigenschappen van rhodopsine worden besproken, die direkt samenhangen met zijn karakter als membraaneiwit. Mogelijkheden om de wisselwerking tussen lipiden en rhodopsine te detekteren en om die wisselwerking te beïnvloeden worden toegelicht en het doel van het onderhavige onderzoek is geformuleerd.

Hoofdstuk 3 beschrijft de materialen en werkwijzen alsmede de verschillende bepalingen die gedurende het gehele onderzoek zijn toegepast. Een methode om rhodopsine in zuivere vorm te isoleren wordt er nader uitgewerkt.

In hoofdstuk 4 wordt gerapporteerd over de lokalisatie van eiwitten in het fotoreceptor membraan. Daarbij hebben we vanzelfsprekend de aandacht vooral gericht op het rhodopsine molekuul, dit temeer omdat het meer dan 85% uitmaakt van al het eiwit in fotoreceptormembranen. Suspensies van deze membranen zijn behandeld met proteolytische enzymen (trypsine, α-chymotrypsine, papaine, subtilisine en pronase).Het blijkt dat alle momenteel bekende eigenschappen van rhodopsine ongevoelig zijn voor deze proteases. Het karakteristieke absorptie spektrum van rhodopsine blijft intakt, alsmede het door belichting teweeg gebrachte patroon van spektrale veranderingen, alsook de thermische gevoeligheid en regeneratie kapaciteit (het vermogen om na belichting en na toevoeging van 11-cis retinaldehyde weer rhodopsine te vormen). Toch kunnen alle gebruikte enzymen,

behalve trypsine, een aanzienlijk deel van rhodopsin (> 25%) afbreken, zoals blijkt uit gel-elektroforetische analyse van de resterende eiwitfragmenten. Wanneer de opbouw van het fotoreceptor membraan wordt verstoord (toevoegen van detergentia, behandeling met fosfolipase C, volledig delipideren van rhodopsine), konstateren we dat de essentiele kenmerken van rhodopsine niet meer bestand zijn tegen proteases, terwijl de eiwitketen vergaand wordt afgebroken. Deze vergelijkende studies tonen aan, dat rhodopsine voor een groot gedeelte door de omgevende lipiden wordt afgeschermd en dat derhalve het eiwit voor een aanzienlijk deel ligt ingebed in het hydrofobe deel van de lipide dubbellaag. Tevens tonen deze experimenten aan, dat voor nagenoeg alle thans bekende eigenschappen van rhodopsine volstaan kan worden met aan substantieel kleiner deel van de eiwitketen.

Het aanbrengen van veranderingen in de micro-omgeving van het eiwit is van belang om te achterhalen in welke mate de eigenschappen van rhodopsine afhankelijk zijn van de aanwezigheid en de samenstelling van de lipide dubbellaag. Dergelijke veranderingen zijn tot stand gebracht door in het fotoreceptor membraan de primaire aminogroepen te blokkeren met een acetyl-groep. Verder is fosfolipase C behandeling van fotoreceptor membranen toegepast, omdat dit enzym de fosfaat esters van de fosfolipiden afsplitst, waardoor diglyceriden achterblijven, die een aanzienlijk minder amfifiel karakter hebben dan de oorspronkelijke fosfolipiden. Vanzelfsprekend betekent ook het volkomen delipideren van rhodopsine met behulp van affiniteitschromatografie een verandering van micro-omgeving. Tenslotte is er nog de mogelijkheid om alle oorspronkelijke lipiden te vervangen door lipiden door middel van een detergens-dialysemethode. Deze procedure leidt ertoe dat rhodopsine weer ingebed wordt in een lipide dubbellaag, mits amfifiele lipiden zijn gebruikt. We kunnen dus de micro-omgeving van het visuele pigment veranderen op verschillende manieren en in verschillende mate, om de invloed hiervan op karakteristieken van rhodopsine te bestuderen.

In hoofdstuk 5 is een van de karakteristieken van rhodopsine in fotoreceptor membranen nader onderzocht, nl. het laatste en langzame deel van de spektrale overgangen die het gevolg zijn van het belichten van rhodopsine.

In hoofstuk 6 zijn de verkregen gegevens gebruikt als referenties om effekten van verandering in de micro-omgeving op het fotolyse gedrag van rhodopsine te achterhalen. Fosfolipase C behandeling van fotoreceptor membranen en ook het volledig delipideren van rhodopsine leiden er toe dat het fotolyse patroon van rhodopsine wordt verstoord, doordat er een blokkering optreedt van één van de spektrale overgangen. Het normale fotolyse patroon van rhodopsine wordt echter hersteld als het pigment weer wordt opgenomen in een lipide bilaag. Daarbij blijkt het van geen enkel belang te zijn wat de aard van het polaire gedeelte der gebruikte amfifiele lipiden is.

In hoofdstuk 7 hebben we de effecten van verandering in de micro-omgeving nader bekeken aan de hand van twee andere katakteristieken van rhodopsine, nl. de thermische gevoeligheid en de regeneratie kapaciteit. Ook aan de hand van deze twee parameters hebben we kunnen konstateren, dat fosfolipase C behandeling, alsook volledige delipidering de eigenschappen van rnodopsine nadelig beïnvloeden. Ook hier wordt een volledig herstel gevonden als rhodopsine weer is opgenomen in een lipide dubbellaag, ongeacht de aard van het polaire gedeelte van de amfifiele lipiden.

In hoofdstuk 8 zijn de resultaten vervat van elektronenmicroskopische studies van fosfolipase C behandelde membranen. We kunnen aan de hand daarvan konkluderen, dat deze behandeling leidt tot het ontstaan van twee-dimensionale (laterale) aggregaten van rhodopsine molekulen.

In hoofdstuk 9 tenslotte, hebben we de resultaten van de voorgaande hoofdstukken integraal besproken en hebben we beargumenteerd, dat de vorming van rhodopsine-aggregaten verantwoordelijk kan worden gesteld voor de nadelige effekten die fosfolipase C behandeling of volledige delipidering op de eigenschappen van rhodopsine hebben. Waarschijnlijk geeft aggregatie aanleiding tot een verstarring van

de eiwitstruktuur en gaat dit niet of in slechts geringe mate gepaard met konformatie-veranderingen in het eiwit. Daaruit hebben we afgeleid dat de enige voorwaarde die rhodopsine aan zijn microomgeving lijkt te stellen is, dat deze het eiwit voldoende flexibiliteit laat. Kennelijk wordt deze voorwaarde vervuld door elk willekeurig amfifiel lipide dat tot nu toe bestudeerd is, mits het rhodopsine molekuul opgenomen is in een dubbellaag van deze lipiden.

Bovenstaande conclusies houden in, dat uit de tot nu toe bekende eigenschappen van rhodopsine niet kan worden afgeleid waarom het natuurlijke fotoreceptor membraan zo'n hoge koncentraties aan onverzadigde lipiden heeft. Mogelijk stelt een nog niet bekende eigenschap van rhodopsine wel deze bijzondere eisen aan de membraansamenstelling. Een voortzetting van de hier aangevangen studies is dan ook van belang. Dit echter niet alleen om het visuele gebeuren beter te leren begrijpen. Door de grote herkenbaarheid van rhodopsine en de eenvoudige wijze waarop op grote schaal fotoreceptormembranen en gezuiverd rhodopsine kunnen worden geïsoleerd, alsmede het gemak waarmee rhodopsine kan worden ingebouwd in kunstmatige membranen, zijn vele studies denkbaar die ook kunnen leiden tot beter begrip van relaties tussen lipiden en eiwitten in andere biomembranen.

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CURRICULUM VITAE

P.J.G.M. van Breugel is op 25 oktober 1947 geboren te Eindhoven. Vanuit zijn woonplaats Sint-Oedenrode bezocht hij het Mgr. Zwijsen College te Veghel waar hij in 1966 het eindexamen HBS-B aflegde. In hetzelfde jaar begon hij met de scheikunde studie aan de Katholieke Universiteit te Nijmegen en behaalde in december 1969 het kandidaatsexamen S_2. In oktober 1972 volgde het doktoraalexamen scheikunde met als hoofdrichting organische chemie; bijvakken: biochemie en biofysische chemie. Sinds 1 september 1972 is hij werkzaam geweest aan de afdeling biochemie van de Medische Faculteit van voornoemde Universiteit, waar het hier beschreven onderzoek is verricht onder leiding van Dr. F.J.M. Daemen en Prof. Dr. S.L. Bonting. Aanvankelijk in dienst van de universiteit, is hij van 1 februari 1973 tot 1 februari 1977 in dienst geweest van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (ZWO) via de Stichting Scheikundig Onderzoek in Nederland (SON).

STELLINGEN

I

Indien fotolyse van rhodopsine gepaard zou gaan met disulfide uitwisseling, biedt het door McDowell en Williams hiervoor gepresenteerde model geen bevredigende verklaring.
J.H.M.McDowell en T.P.Williams, Vision Res. 16 (1976) 643-646

II

Het door Reich en Emrich gelegde verband tussen fotolyse van rhodopsine en beweging van calcium ionen kan niet worden ontleend aan hun experimenten.
H.M.Emrich en R.Reich, Pflügers Arch. 364 (1976) 17-21
R.Reich en H.M.Emrich, Pflügers Arch. 364 (1976) 23-28 .

III

De door Whikehart en Hess gevolgde procedure voor het bereiden van opsine-bevattende membranen maakt een zinvolle interpretatie van hun experimenten onmogelijk.
D.R.Whikehart en H.H.Hess, Exp.Eye Res. 24 (1977) 279-289

IV

Bij de toepassing van monofunktionele imidaten voor modifikatie van eiwitten dient rekening te worden gehouden met het ontstaan van inter- en intra-molekulaire "cross-links".
D.T.Browne en S.B.H.Kent, Biochem. Biophys. Res. Commun. 67 (1975) 126-132

V

De konklusie van Konturek en medewerkers, dat bombesine geen direkt effekt heeft op de exokriene pankreas wordt niet door hun experimenten gerechtvaardigd.
S.J.Konturek, R.Król en J.Tasler, J. Physiol. 257 (1976) 663-672
M.Deschodt-Lanckman, P.Robberecht, P.de Neef, M.Lammens en J.Christophe, J. Clin. Invest. 58 (1976) 891-898

VI

Zolang biologische aktiviteit en molekulaire ordening in het komplex tussen het apoproteine van cytochroom C en het hemine-bevattende broomcyaan fragment niet zijn aangetoond, is de door Hantgan en Taniuchi voorgestelde struktuur spekulatief.
R.R.Hantgan en H.Taniuchi, J. Biol. Chem. 252 (1977) 1367-1374

VII

De konventie om de hoofdassen van een Elektrisch Veld Gradient-tensor zo te kiezen, dat $|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|$ dient vervangen te worden door een konventie waarbij $V_{zz}>0$ en $0 \leq \eta < 3$ ($\eta = (V_{xx}-V_{yy})/V_{zz}$).

VIII

Het verdient aanbeveling om vroegtijdig in de universitaire chemie studie een kursus op te nemen over veiligheidsaspekten, waaronder het verantwoord verwerken van afvalprodukten.

IX

De huidige enquêteringslust zou vergaan, indien statistisch werd weergegeven hoe vaak verzamelde gegevens leiden tot statistisch irrelevante statistieken.

X

Bij de huidige situatie van de arbeidsmarkt is de term "wegpromoveren" nergens meer van toepassing dan bij de universitaire promotie, zodat het aan te bevelen is om fondsen voor promotieonderzoek te gaan gebruiken voor het omscholen van gepromoveerden.

Nijmegen, 3 juni 1977 P.J.G.M. van Breugel